Identification and Characterization of A Novel *Salmonella* Gene Product, STM0029, which Contributes to the Resistance to Host Antimicrobial Peptide Killing

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M.Sc. Mikrobiologie, Heng-Chang Chen

Präsidentin/Präsident der Humboldt-Universität zu Berlin

Prof. Dr. Jan-Hendrik Olbertz

Dekanin/Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I

Prof. Dr. rer. nat. Andreas Herrmann

Gutachter/innen: 1. **Prof. Dr. Richard Lucius**

2. **Univ.-Prof. Dr. Lothar H. Wieler**

3. **Prof. Dr. Thomas Eitinger**

4. **Priv.-Doz. Dr. Klaus Heuner**

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For my parents

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ABSTRACT

Salmonella spp. are facultative intracellular pathogens, which cause gastrointestinal and systemic diseases in a broad range of hosts including animals and humans. In addition to virulence genes clustered within pathogenicity islands, numerous additional genes scattered throughout the genome are also involved in various aspects of *Salmoenlla* virulence and pathogenesis. In this study, I identified a *Salmonella* putative transcriptional regulator encoded by a previously uncharacterized open reading frame designated STM0029. Deletion of STM0029 altered the expression of genes involved in both the resistance to host bactericidal challenges, and bacterial cell wall biosynthesis in *S*. Tyhpimurium.

The ΔSTM0029 strain showed a defect in the resistance to host antimicrobial peptides, including α-defensin-1, β-defensin-1, β-defensin-2, LL-37, and polymyxin B as well as serum challenges compared to the wildtype. Unexpectedly, expression of STM0029 was found to be repressed by the PmrA/B two component system, but appeared to be independent of the PhoP/Q two component system, both of which are well-known regulatory systems involved in the regulation of expression of genes involved in *Salmonella* intracellular survival. Notably, the expression of a set of genes involved in bacterial LPS O-antigen and peptidoglycan biosyntheses and modifications showed decreases in the absence of STM0029. These experimental results indicate that the STM0029 gene product in *S*. Typhimurium contributes to resistance against host cell defense mechanisms, likely through regulation of genes involved in LPS O-antigen and peptidoglycan biosynthesis and modifications.

Key words

Salmonella/Antimicrobial peptide/PmrA/B and PhoP/Q two component system/LPS O-antigen

ZUSAMMENFASSUNG

Salmonella spp. sind fakultative intrazelluläre Pathogene, die gastrointestinale und systemische Erkrankungen in einem umfassenden Wirtsbereich, einschließlich Tier und Mensch, hervorrufen. *Salmonella* benötigt verschiedene Virulenzgene für die Infektion welche auf sogenannten Salmonella Pathogenitäts-Inseln (SPI) kodiert sind. Hinzu kommt, dass auch zahlreiche im *Salmonella* Genom verstreuten Gene an verschiedenen Aspekten von Virulenz und Pathogenese beteiligt sind. In der vorliegenden Studie wurde die Funktion eines zuvor nicht beschriebenen putativen transkriptionellen Regulators (STM0029) charakterisiert und definiert. Dieser scheint für die Abwehr von zellulären bakterizid wirkenden Verbindungen und das Überleben des Bakteriums innerhalb einer intrazellulären Nische von entscheidender Bedeutung zu sein.

Die STM0029-deletierte Mutante wies eine gesteigerte Sensitivität gegenüber antimikrobiellen Peptiden und bakteriziden Verbindungen auf. Dazu zählten α-Defensin-1, β-Defensin-1, β-Defensin-2, LL-37 und Polymyxin B sowie Komponenten des Komplementsystems. Unerwartet war die Beobachtung, dass die Expression von STM0029 durch das PmrA/B Zwei Komponenten System reprimiert vorlag, während das PhoP/Q Zwei Komponenten System keinen Einfluss auf die Expression von STM0029 zu scheinen hat. Beide Komponent Systeme spielen bekanntlich eine entscheidende Rolle bei der Expressionsregulation von Genen die für das intrazelluläre Überleben von *Salmonella* wichtig sind. Bemerkenswert ist, dass ein Set von Genen welche an der Biosynthese und/oder der Modifikation für das LPS O-Antigen sowie des Peptidoglykans in der bakteriellen Zellwand beteiligt ist, im STM0029 Deletionshintergrund herab reguliert vorlag. Dieses Ergebnis deutet darauf hin, dass das STM0029 Genprodukt die Persistenz des Pathogen in Wirtszellen beeinflusst. Möglicherweise geschieht dies durch das Umgehen von wirtseigenen Abwehrmechanismen.

Schlüsselbegriffe

Salmonella/Antimikrobielle Peptide/PmrA/B und PhoP/Q Zwei Komponenten Systeme/LPS O-Antigen

ABBREVIATIONS

ACL	Antigen carrier lipid
ACP	*R*-3-hydroxymyristoyl-acyl carrier protein
AMP	Antimicrobial peptide
APS	Ammonium peroxidisulfate
Ara4N	4-amino-4-deoxy-L-arabinose
bp	Base pairs
BCA	2,2' Bicinchoninic acid
BSA	Bovine serum albumin (Fraction V)
ºC	Celsius
cDNA	Complementary DNA
CFU	Colony forming units
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamino tetraacetic acid
EGTA	Ethylene-glycol-bis (2-aminoethyl) tetraacetic acid
FCS	Fetal Calf Serum
Fig	Figure
GFP	Green fluorescent protein
IPTG	Isopropyl-thio-ß-D-galactopyranoside
kb	Kilo base
Km	Kanamycin
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
m	Milli
M	Molar
µ	Micro
m-DAP	*meso*-diaminopimelic acid
MurNAc	*N*-acetylmuramic acid
OD	Optical density
ONPG	o-Nitrophenyl-ß-D-galactopyranoside
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pEtN	Phosphoethanolamine
PG	Peptidoglycan
Pi	Inorganic phosphate
PMA	Phorbol 12-myristate 13-acetate
ppGpp	Guanosine tetraphosphate
PRR	Pattern-recognition receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute

RT	Room temperature
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCI	*Salmonella* chromosomal island
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecylsulfate
SPI	*Salmonella* pathogenicity island
T3SS	Type 3 secretion system
TBE	Tris-borate-EDTA
TCS	Two component system
TEMED	N,N,N',N' - Tetramethylethylenediamine
TIR	Toll/Interleukin-1receptor
TLR	Toll-like receptor
X-gal	5-Bromo-4-chloro-3-indoxyl- β -D-galactoside

1. INTRODUCTION

1.1. *Salmonella* and Salmonellosis

Salmonella is a Gram-negative, motile, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. Classification of *Salmonella* based on serology and phage susceptibility assays has currently identified more than 2500 serovars. The *Salmonella* genus contains two species, *enterica* and *bongori* (Brenner et al., 2000). Based on the variation in their biotypes, *S. enterica* is further divided into six subspecies, including *enterica* (group I), *salamae* (group II), *arizonae* (group IIIa), *diarizonae* (group IIIb), *houtenae* (group IV), and *indica* (group VI) containing 2443 serovars (Le Minor & Popoff, 1987; reviewed by Tindall et al., 2005). Most of the Salmonellae that cause disease are in the subspecies *Salmonella enterica* subspecies *enteria* (Mastroeni and Maskell, Book Chapter 1, 2006). *Salmonella* causes a wide range of diseases in human and animal hosts ranging from a self-limiting enteritis to systemic infections, depending on *Salmonella* serotypes as well as the host and immune status (Marmion et al., 1953; reviewed by Coburn et al., 2007). While the serovars Typhi and Paratyphi are strict human pathogens (Hornick et al., 1970; Thong et al., 1994), *Salmonella enterica* serovars Typhimurium and Enteritidis are broad host-range pathogens, infecting both human and animal hosts (Mastroeni and Maskell, Book Chapter 3, 2006). Over the last 25 years, some of the most commonly detected serovars from chickens, including *S*. Enteritidis and *S*. Heidelberg are also among the top five serovars associated with human infections (Centers for Disease Control and Prevention, 2008; Foley et al., 2008).

Salmonella enterica is a food-borne pathogen of continuing global significance. The strict human-adapted serovars, *S*. Typhi and *S*. Paratyphi, cause typhoid fever characterized by fever, abdominal pain and transient diarrhoea. It has been estimated that there are approximately 21 million cases with a mortality of 1% (Crump et al., 2004). Non-typhoid *Salmonella* infections in humans usually result from consumption of contaminated food and water and causes a self-limiting gastroenteritis. As reported in 1999, in the USA alone there were estimated 1.4 million cases of non-typhoid *Salmonella* infections annually, resulting in approximately 600 deaths (Mead et al., 1999). In Germany, more than 80% of the human isolates from the cases reported to the Enteric Reference Centre at the Robert Koch Institute in 1995 were comprised of serovar Enteriditis (61.3%) and serovar Typhimurium (23.4%) (Rabsch et al., 2001). Worldwide estimates of cases of non-typhoid *Salmonella* infection range from 200 million to 1.3 billion, with an estimated death toll of 3 million each year (Hohmann, 2001).

Human salmonellosis is typically associated with the consumption of contaminated foods, such as fresh and processed meat and poultry, eggs, and fresh products (Benenson and Chin, 1995; Mead et al., 1999; Tauxe, 1991). Symptoms of salmonellosis include diarrhea, fever, vomiting, and abdominal cramps (McGhie et al., 2009). Intermittent shedding of the pathogen by domestic animals is thought to provide a constant reservoir for infection and contamination of food. Salmonellosis in domestic animal species is also important for public health as the major reservoir and source of food-borne human infections. Industrialisation and large scale food distribution, increased consumption of raw or slightly cooked foods, an increase in immuno-compromised patient populations, deteriorated public infrastructure and evolution of multi-drug-resistant *Salmonella* have been all proposed as possible reasons for the steady increase in the incidence of *Salmonella* infections (Darwin and Miller, 1999a).

1.2. *Salmonella* host cell infection

Salmonella enterica species are facultative intracellular pathogens that infect and replicate within non-phagocytic epithelial cells, enterocytes, hepatocytes as well as professional phagocytic macrophages. Carter and Collins (1974) suggested that there are two major sites of infection in the intestinal lumen: Peyer's patches and the epithelium covering the villi, both of which are important for *Salmonella* invasion of the intestinal wall. Peyer's patches and solitary intestinal lymphoid tissues are sites of organized lymphoid structures in the distal ileum. *Salmonella* can pass through the epithelium through different mechanisms. *Salmonella* can traverse the barrier either through M cells in the follicle-associated epithelium (FAE) or epithelial cells forming the FAE (Tam et al., 2008). Following invasion of non-phagocytic host cells, *Salmonella* survives and replicates within a modified phagosome known as the *Salmonella*-containing vacuole (SCV) (Fig. 1, see page 15). Successful invasion and further infection processes of *Salmonella* in host cells are mediated by chromosomally-encoded virulence genes, which are clustered on *Salmonella* pathogenicity islands (SPIs; Fitts, 1985; Groisman et al., 1993; Groisman and Ochman, 1997; reviewed by McGhie et al., 2009). Two major pathogenicity islands, SPI1 and SPI2 encoding type III secretion systems (T3SS), are required for invasion and SCV biogenesis/ intracellular survival, respectively. During invasion, SPI1-encoded virulence genes manipulate host cell processes such as actin polymerisation, membrane trafficking and signal transduction (reviewed by McGhie et al., 2009; reviewed by Steele-Mortimer, 2008); whereas SPI2-encoded genes are expressed only after *Salmonella* entry into host cells and alters the maturation of the SCV and promotes intracellular replication (Hensel et al., 1998).

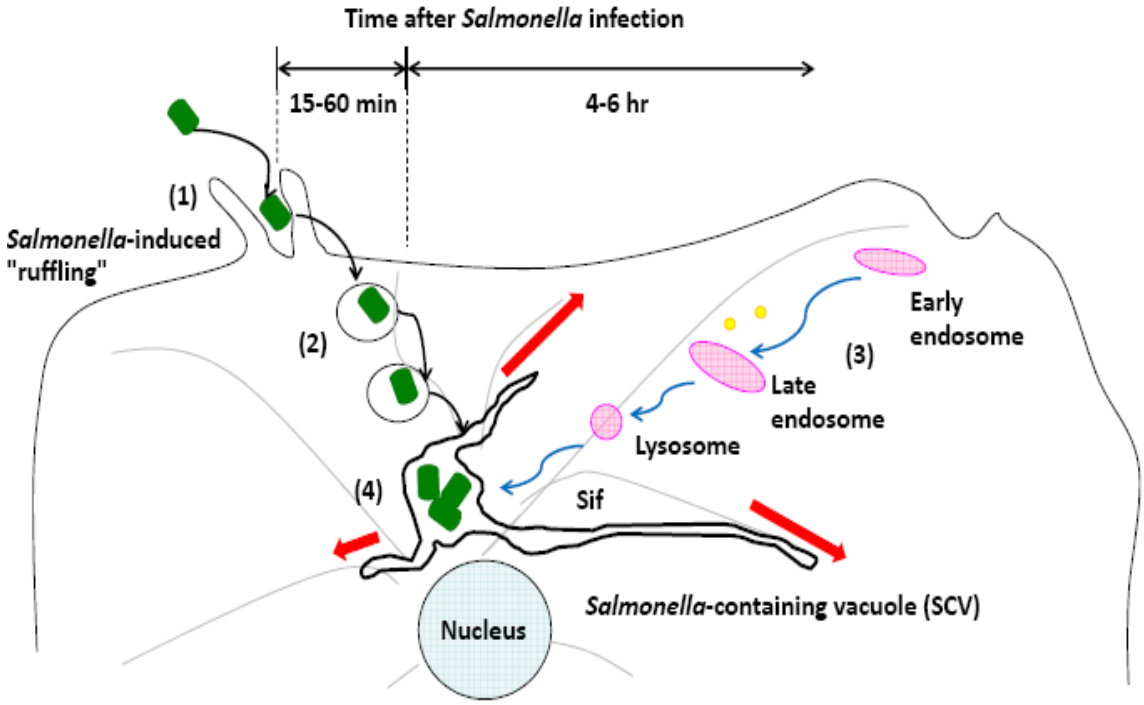


Fig. 1. Schematic representation of *Salmonella* infection. *Salmonella* invades host cells by inducing actin polymerization seen as membrane ruffles on the plasma membrane (1). After infecting host cells, *Salmonella* is internalised into the early SCV, within which *Salmonella* is able to survive and replicate (2). Within 15–60 min of post-infection, early endosomal proteins are replaced by proteins normally found on late endosomes or lysosomes and SCVs are predominantly localised near the nucleus (3). Within 4-6 hr of post-infection, *Salmonella* replication is initiated and SCV tubules (Sifs)* extend radially along microtubules (4). The movement and localisation of these cell compartments are mediated largely by interactions with microtubule-based motors such as dynein and kinesin, which mediate retrograde (blue arrows) and anterograde movement (red arrows), respectively. *Salmonella* utilises the same cellular system for translocation of SCVs towards the nucleus and subsequent extension of Sifs. The figure is modified from Steele-Mortimer, 2008.

*Sifs: *Salmonella*-induced filaments (Sifs) are tubular structures utilised by *Salmonella* to elongate the vacuole, within which it resides (Brumell et al., 2001).

1.3. *Salmonella* virulence factors

Approximately 200 genes or 4% of the *S.* Typhimurium genome are considered to be virulence factors (Bowe et al., 1998). While these virulence genes can be found scattered throughout the *Salmonella* chromosome, analysis of the genetic structure reveals that these genes often cluster in regions referred to as *Salmonella* pathogenicity islands (SPIs) (Groisman and Ochman, 1997; reviewed by McGhie et al., 2009). Pathogenicity islands have also been described in many Gram-negative animal and plant pathogenic bacteria. Examples

include the locus of enterocyte effacement (LEE) region of enterohemorrhagic *Escherichia coli* (Elliot et al., 1998); the Mix/Spa region of *Shigella* spp. (reviewed in Hueck, 1998); the Exs/Psc region of *Pseudomonas aeruginosa* (reviewed by Hueck, 1998); and the Hrc/Hrp region of *Ralstonia solonacearum* (Van Gijsegem et al., 1993). In *Salmonella*, these islands contain multiple functionally related genes, which are necessary for a specific virulence phenotypes and in general have a lower GC content compared to the rest of the bacterial genome, supporting the idea that these pathogenicity islands were horizontally acquired (McClelland et al., 2001). To date, at least 12 SPIs have been characterised in *Salmonella*, of which five SPIs have been most studied (Hensel, 2004).

1.3.1. *Salmonella* Pathogenicity Island 1 (SPI1)

Salmonella pathogenicity island 1 (SPI1) is a 40-kb region located at centosome 63 on *Salmonella* chromosome flanked by *flhA* (STM1913) and *mutS* (STM2909) (Mills et al., 1995; Hensel, 2004). Based on sequence and hybridization data, SPI1 is highly conserved and has been reported to be present in *S. bongori* and all subspecies of *Salmonella enterica* (Ochman and Groisman, 1996). Unlike the majority of the pathogenicity islands, SPI1 is not associated with a tRNA gene. SPI1, which harbours at least 35 genes, encodes a type III secretion system (T3SS) and associated secreted effector proteins (Kimbrough and Miller, 2002). A number of reports indicate that many SPI1-encoded genes are involved in mediating macrophage apoptosis *in vitro* (Chen et al., 1996) and trigger enteritis and intestinal inflammation (Galyov et al., 1997; Wallis and Galyov, 2000).

The main regulators encoded within SPI1 participating in *Salmonella* invasion include HilA, HilC, HilD, InvF and SprB (Bajaj et al., 1995; Darwin and Miller, 1999b; Eichelberg and Galán, 1999; Lostoh et al., 2000; Rakeman et al., 1999; Schechter et al., 1999). HilA plays a central role as a major transcriptional regulator to activate the expression of *S.* Typhimurium invasion genes (Bajaj et al., 1995). Expression of the genes within SPI1 and multiple loci encoded outside of SPI1 are under the control of the local regulator InvF. Currently, 12 *Salmonella* serovar Typhimurium effector proteins, which are transported *via* the SPI1 T3SS have been identified (Galan, 2001). The gene products encoding the T3SS include those for the core apparatus (SpaO, SpaP, SpaQ, SpaR, SpaS, InvA, InvC and OrgB), needle complex components (PrgH, PrgI, PrgJ, PrgK, InvG and InvJ) and translocon (SipB, SipC and SipD) (Collazo and Galán, 1997; Kimbrough and Miller, 2002). Many additional effector proteins, including SopE, SopE2, SopA, SopB/SigD, SopD, SlrP, and SspH1 are encoded elsewhere in the chromosome (Bakshi et al., 2000; Jones et al., 1998; Miao et al.,

1999; Wood et al., 1996; Wood et al., 1998; Wood et al., 2000). Zhang et al. (2002) reported that SipA, SopA, SopB, SopD, and SopE2 are important for *S.* Typhimurium to induce diarrhea in calves. InvB (Bronstein et al., 2000), SicA (Tucker and Galan, 2000) and SicP (Fu and Galan, 1998) encode the main chaperones protecting secreted proteins during translocation.

1.3.2. *Salmonella* Pathogenicity Island 2 (SPI2)

Salmonella pathogenicity island 2 (SPI2) is a 40-kb region mapped to 31 minutes on the chromosome and associated with the *valV* tRNA. Of the 40-kb sequence, only a region of 15-kb is present in both *S. enterica* and *S. bongori* while the remaining 25-kb region, harbouring genes encoding a second T3SS, is restricted to subspecies of *S. enterica* and is required for the systemic virulence. Recent studies indicate that the function of the T3SS encoded within SPI2 is central for the ability of *S. enterica* to cause systemic infections and for intracellular proliferation (Hensel et al., 1998). Genes encoding of the SPI2 regulatory proteins have been given designations related to their functions, and are termed *ssr* (secretion system regulator), *ssa* (secretion system apparatus), *sse* (secretion system effector) and *ssc* (secretion system chaperone), respectively.

1.3.3. *Salmonella* Pathogenicity Island 3 (SPI3)

Salmonella pathogenicity island 3 (SPI3) is a 17-kb locus, which has been horizontally acquired and is present as an insertion in the *selC* tRNA locus. Although it is conserved in both *S.* Typhi and *S.* Typhimurium, there are large variations in other subspecies. SPI3 harbours 10 open reading frames organised in six transcriptional units, which include the *mgtCB* operon encoding the macrophage survival protein MgtC and the Mg^{2+} transporter MgtB (Blanc-Potard et al., 1999). Interestingly, MgtC, which is encoded by a *Salmonella*-specific gene, is required for intracellular survival within macrophages, virulence in mice, and growth in low-Mg^{2+} media. The *mgtC* gene is transcriptionally controlled by the PhoP/Q regulatory system, which is involved in the adaptation to a low-Mg^{2+} environment and is a major regulator of virulence functions in *Salmonella* (Alix and Blanc-Potard, 2007) (see section 1.3.8.1. for further details, p. 19).

1.3.4. *Salmonella* Pathogenicity Island 4 (SPI4)

Salmonella pathogenicity island 4 (SPI4) is a 27-kb region that carries six genes designated *siiABCDEF*. Unlike SPI1 and SPI2, the genes including *siiC*, *siiD* and *siiF* encode a type I

secretion system (T1SS) for the secretion of SiiE, a large (~600 kDa) protein contributing to the colonization of the bovine intestine (Gerlach et al., 2007; Kiss et al., 2007). SiiE has been demonstrated to function as a large, non-fimbrial adhesin to mediate *Salmonella* adhesion to epithelial cells (Gerlach et al., 2007). The role of SPI4 remains unclear, but a recent study using signature-tagged mutagenesis identified SPI4 as a colonization factor during infection of calves (Morgan et al., 2007). Lawley et al., (2006) identified a strain defective in SPI4 genes as being attenuated in a model for long-term persistence.

1.3.5. *Salmonella* Pathogenicity Island 5 (SPI5)

Salmonella pathogenicity island 5 (SPI5) is a 7.6-kb insertion within the *serT-copS*/*R* intergenic region. This region appears to be conserved in *Salmonella* serovars; however, it is absent in other pathogenic bacteria. SPI5 also encodes *pipABCD* and *orfX* in addition to *sopB*/*sigD*, which have been identified previously (Wood et al., 1998). SopB/SigD is secreted through the SPI1 T3SS and has been shown to be involved in actin rearrangement, phagocytosis, biosynthesis of SCV, fluid secretion and diarrhoeal symptoms. Similar to the activity of SopB, *S.* Dublin *pip* mutants were attenuated for their enteropathogenicity in calf ileal loop models (Wood et al., 1998).

1.3.6. Other *Salmonella* pathogenicity islands

Besides the five major pathogenicity islands as mentioned above, different serovars of *Salmonella* spp also hold other pathogenicity islands, which still have not been well studied. For example, the *safABCD* operon is located within *Salmonella enterica* centisome seven genomic island (SCI), also known as SPI6 in *S.* Typhi (Folkesson et al., 2002; Parkhill et al., 2001). This operon encodes *Salmonella*-specific putative atypical fimbriae; however, the *S.* Typhimurium *saf* operon was not required for virulence in mice (Folkesson et al., 1999; Morgan et al., 2004). SPI7, which is also referred to as the major pathogenicity island (MPI) of serovar Typhi, harbours genes involved in expression of the Vi antigen, a capsular exopolysaccharide (Hacker et al., 1997; Liu and Sanderson, 1995).

1.3.7. Plasmid-encoded virulence genes

Plasmids of *Salmonella* spp vary in size from 2- to more than 200-kb. The best-described group of plasmids are virulence plasmids (50 - 100-kb in size), which are present in serovars Enteritidis, Typhimurium, Dublin, Choleraesuis, Gallinarum, Pullorum and Abortusovis. These large virulence plasmids are involved in *Salmonella* systemic infections and increase

the growth rate of the bacteria at sites beyond the intestine in mice models (Gulig, 1990; Guling and Doyle, 1993). One common region conserved among virulence plasmids is the 7.8-kb region *spvRABCD* operon. SpvR is a positive transcriptional regulator of the *spvABCD* operon. Signals for expression of the *spv* locus are growth restriction, reduced nutrient supply or low pH (Guiney et al., 1995). SpvB and SpvC are the central effector genes of this operon, since the presence of *spvBC* genes alone is sufficient to complement for the missing virulence plasmid in *S*. Typhimurium (Matsui et al., 2001).

In addition to the *spv* virulence plasmid, other plasmids, which are present in some, but not all other serovars, harbour additional virulence-related genes such as *rck* (resistance to complement killing) (Ho et al., 2010), *pef* (plasmid-encoded fimbriae), *srgA* (SdiA-regulated gene, putative disulphide bond oxidoreductase) (Jarrott et al., 2010) and *mig*-5 (macrophage-inducible gene coding for putative carbonic anhydrase).

1.3.8. Regulation of *Salmonella* virulence genes

The ability of an organism to orchestrate responses to environmental changes depends on transcriptional regulatory factors and networks used to control expressions of multiple genes. Currently, many regulators which are important to this response have been reported: the two component systems (TCSs) PhoP/Q, PmrA/B, OmpR/EnvZ; the alternative sigma factors RpoS and RpoE; and signal molecules such as cyclic adenosine monophosphate (cAMP), cyclic diguanosine monophosphate (diGMP), diadenosine tetraphosphate (Ap4), and guanosine penta- and tetraphosphate ((p)ppGpp) (De Las Penas et al., 1997b ; Fang et al., 1996; Groisman et al., 1989; Gunn, 2008; Pizarro-Cerdá and Tedin, 2004; Prost and Miller, 2008).

1.3.8.1. The PhoP/Q two component system

S. Typhimurium has known to have three periplasmic phosphatases: an acid hexose phosphatase, a cyclic 2',3'-nucleotide phosphodiesterase and a non-specific acid phosphatase (Kier et al., 1977). The function of PhoP in *Salmonella* was originally found to regulate the locus encoding non-specific acid phosphatase (Kier et al., 1978). Later, the *phoP* mutant (designated MS7953s by Fields et al., 1986) identified by Tn*10* insertional transposon mutagenesis also showed a defensin-sensitive phenotype and a defect in intracellular survival within macrophages (Fields et al., 1986; Fields et al., 1989). The PhoP/Q TCS is composed of a membrane-bound sensor histidine kinase PhoQ and the cytosolic response regulator PhoP. Miller et al. (1989) first reported that PhoQ serves as a sensor kinase for PhoP response

regulator. PhoQ has been sequenced and found to encode a membrane protein with homology with the C-terminal kinase domains of a sensor kinase, EnvZ, which belongs to the *Salmonella* OmpR/EnvZ two component system (Miller, et al., 1989) (see section 1.3.8.3. OmpR/EnvZ for further details, p. 22). Activation of the PhoQ sensor kinase in response to specific environmental signals leads to *trans*-autophosphorylation of PhoQ and subsequently transfers phosphate to PhoP. Phosphorylated PhoP is able to control expression of a number of PhoP-activated genes (*pag*) and PhoP-repressed genes (*prg*) (Perez et al., 2009). To date, more than 200 *pag* and *prg* genes have already been identified in *S.* Typhimurium through microarray analyses and motifs sceening (Monsieurs et al., 2005). The major *pag* gene products whose functions have been defined include PagA, PagB, PagC, PagD, PagJ, PagK and PagM. *pagC* is the first gene to be reported that its expression is required PhoP (Miller et al., 1989). PagC (188 amino acid long), which encodes a membrane protein is similar to the ail-encoded eucaryotic cell invasion protein of *Yersinia enterocolitica* and the lom-encoded protein of bacteriophage lambda (Pulkkinen and Miller, 1991). In addition, expression levels of *pagA* and *pagB* have also been shown increases inside macrophages (Alpuche-Aranda et al., 1992). Moreover, Belden and Miller (1994) reported that mutations in *pagD*, *pagJ*, *pagK*, and *pagM* were significantly attenuated for mouse virulence. PagP, which encodes an outer membrane palmitoyl transferase is able to catalyze the addition of palmitate to lipid A by transfer of fatty acid from the inner leaflet of the outer membrane of lipid A (Guo et al., 1998).

To date, functions of the PhoP/Q TCS have been reported to be involved in many aspects of *Salmonella* pathogenesis, including the Mg^{2+} transporter (García Véscovi et al., 1996; Soncini et al., 1996), survival within macrophages (Fields et al., 1986; Groisman and Saler, 1990), modifications of lipopolysaccharide (LPS) (Mouslim and Groisman, 2003) (see section 1.5.2.2. for further details, p. 32), and actives a further two component system, *e.g.* the PmrA/B TCS (Groisman, 2001; Kato et al., 2007; Kox et al., 2000) (see section 1.3.8.2. for further details).

1.3.8.2. The PmrA/B two component system

The *pmrA* locus was originally isolated by selection of mutants growing on plates containing various concentrations of Colistin E sulfate (Mäkelä et al., 1978). Colistin (polymyxin E) discovered in 1949 is a compound belonging to polymyxin antibiotics and is non-ribosomally synthesized by *Bacillus polymyxa* subspecies *colistinus* Koyama (reviewed by Falagas and Kasiakou, 2005; Komura and Kurahashi, 1979; Koyama et al., 1950). Roland et al. (1993)

further identified and sequenced this *pmrA* locus and found that this locus encodes the *pmrA* gene, which contributes to the resistance to polymyxin B and the neutrophil peptides CAP37 (azurocidin) and CAP57 (bactericidal permeability-increasing protein) challenges. Later, expression of the PmrA response regulator was found to be activated by Fe^{3+}, which is a signal sensed by the PmrB sensor histidine kinase (Wosten et al., 2000). The PmrA/B TCS is also indirectly activated by Mg^{2+}, which is involved in a pathway that requires the Mg^{2+}-responsive PhoP/Q TCS *via* the connection by the PhoP-activated PmrD protein (Kox et al., 2000; Kato and Groisman, 2004; Kato et al., 2007).

Vaara et al. (1981) reported that the *pmrA* mutant showed an increased level of substitution by 4-amino-4-deoxy-L-arabinose (Ara4N) at bacterial lipopolysaccharide (LPS) lipid A phosphoryl groups. To date, many of the characterized PmrA-regulated genes are believed to participate in the modification of LPS (see section 1.5.2.2.2.1. for further details, p. 35). These modifications include the addition of 4-amino-4-deoxy-L-arabinose and phosphoethanolamine (pEtN) to bacterial LPS lipid A and the LPS core region (Heptose I). Ara4N addition occurs on the outer surface of the inner membrane before LPS is flipped outside of the outer membrane. According to microarray studies, mutagenesis and *in silico* analysis (Marchal et al., 2004), the PmrA/B TCS has been found to regulate up to 100 genes in *Salmonella,* of which many of the PmrA/B-associated genes are involved in LPS modifications. The well-studied PmrA-regulated genes include *pmrF* (*pbgP* or *arn*) (Gunn et al., 1998; Trent et al., 2001), *pmrC* (Lee et al., 2004), *pmrE* (Gunn et al., 1998; Zhou et al., 2001) and *cptA* (Tamayo et al., 2005). PmrF, which encodes an inner membrane enzyme utilises the novel lipid undecaprenyl phosphate-α-L-Ara4N as its sugar donor and transfers L-Ara4N to lipid A (Trent et al., 2001). PmrC mediates the addition of pEtN to the 1-phosphate of LPS lipid A (Lee et al., 2004; Tamayo et al., 2005). PmrE (Ugd/PagA), which encodes an UDP-glucose dehydrogenase is necessary to produce aminoarabinose or for the addition of aminoarabinose to LPS lipid A (Gunn et al., 1998). CptA is required for the addition of pEtN to the LPS core region; however, mice infected by the intraperitoneal or oral routes with a mutation of *cptA* showed no differences in the 50% lethal dose (Tamayo et al., 2005). It has also been reported that some of the PmrA/B-regulated downstream genes, *e.g.* those encoding PmrE and Wzz, which determine the LPS O-antigen chain length, are also regulated by another *Salmonella* two component system, the RcsC/YojN/RcsB TCS (Delgado et al., 2006). The RcsC/YojN/RcsB TCS is composed of the sensor RcsC, the response regulator RcsB and the histidine-containing phosphotransfer protein YojN, which is used as a mediator in the phosphoryl transfer from RcsC to RcsB (Majdalani and Gottesman, 2005). The Rcs system in

Salmonella spp is activated by a mutation of *igaA* (Tierrez and Garcia-del Portillo, 2004) or *tolB* (Mouslim et al., 2003) as well as growth under conditions of low Mg^{2+} and Fe^{3+} (Mouslim and Groisman, 2003). However, not all PmrA-regulated genes are involved in LPS modifications, *e.g. dgoA*, *yibD*, *aroQ*, *mig-13* and *sseJ* (Gunn, 2008).

Orthologs of the *Salmonella* PmrA/B TCS exist in several Gram-negative pathogens, *e.g. Yersinia spp.* (Flamez et al., 2007), *Escherichia coli* (Raetz, 2001) and *Pseudomonas aeruginosa* (Moskowitz et al., 2004). Modifications of LPS mediated by the PmrA/B TCS result in the resistance to host antimicrobial peptides (AMP) killing subsequently. In both *Legionella pneumophila* and *Coxiella burnetii*, orthologs of the PmrA/B TCS, which have been described to control the *icm*/*dot* type IV secretion pathway are essential for the transfer of virulence effectors into host cells (Zusman et al., 2007).

1.3.8.3. The OmpR/EnvZ two component system

The EnvZ/OmpR system is one of the ancestral two component system used by various bacteria, including *Escherichia coli* (Kanamaru et al., 1989), *Salmonella* spp (Lindgren et al., 1996) and *Shigella* spp (Bernardini et al., 1990). This two component system mediates signal transduction in response to environmental osmolarity changes in *Escherichia coli* (Kanamaru et al., 1989; Yang and Inouye, 1991). The histidine kinase EnvZ, which consists of 450 amino acid residues exists as a dimer located in the inner cytoplasmic membrane of *E. coli*. EnvZ undergoes trans-autophosphorylation on the highly conserved Histidine-243 residue (Yang and Inouye, 1993) and regulates the phosphorylation state of the transcriptional response regulator OmpR to respond to the osmolarity changes in the environment (Cai and Inouye, 2002). The phosphoryl group is further subsequently transferred to the conserved Asparagine-55 residue of OmpR (Yang and Inouye, 1993). Phosphorylated OmpR therefore serves as a transcription factor differentially to modulate expressions of major outer membrane porins encoded by *ompC* and *ompF* (Nikaido and Vaara, 1985; reviewed by Nikaido, 2003). The OmpC and OmpF products are able to form channels in the outer membrane, which allow diffusion of small hydrophilic molecules of less than 650 Dalton (Nikaido and Vaara, 1985). In *Salmonella* spp, both OmpR/EnvZ and PhoP/Q TCSs participate in the regulation of the expression with SPI2 genes as well (Feng et al., 2003; Kim and Falkow, 2004). OmpR is able to bind to the promoter and induces expression of the SpiR/SsrB TCS encoded in SPI2 in order to activate SPI2 genes; PhoP directly binds to the *ssrB* promoter and induces *spiR* transcription indirectly by inducing transcriptional activation of the transcription factor SlyA (Fass and Groisman, 2009).

1.3.8.4. Alternative sigma factors RpoS and RpoE

In *Escherichia coli*, RNA polymerase consists of the subunits of α_2, β, β', and a specific σ. A sigma factor (σ factor) is a prokaryotic transcription initiation factor that enables specific binding of RNA polymerase to gene promoters (reviewed by Gruber and Gross, 2003). RpoS (σ^{38}), which is an alternative sigma factor functions in many aspects of bacterial physiologies, including tolerance of environmental stress challenges (osmotic, oxidative stress and acid shock) and the growth transition to stationary phase (Hengge R, 2008; McMeechan et al., 2007). Expression of RpoS is regulated by complex mechanisms combining with multiple stimulations at transcription, translation and protein stability levels (Hengge-Aronis, 2002). In *Salmonella* spp, RpoS is also involved in the regulation of *Salmonella*-encoded virulence plasmids (*spvRABCD* genes) (Fang et al., 1992; Norel et al., 1992) and *Salmonella* infections as well (Nickerson and Curtiss, 1997).

The *rpoE* gene encodes the σ^E transcription factor, which positively regulates the envelope-stress response to extracytoplasmic unfolded proteins (De Las Penas et al., 1997). The σ^E factor is originally identified as a heat shock factor (Rouviere et al., 1995) and is essential for *E. coli* growth at high temperature (Hiratsu et al., 1995). In *E. coli*, the σ^E factor up- and down-reguates as many as 200 genes involved in the field of metabolism during its expression at either the exponential or the stationary phase (Kabir et al., 2004; Rhodius et al., 2006). In *Salmonella* spp, functions of RpoE have been reported to be involved in the activation of expressions of flagellar genes under hyperosmotic stress (Du et al., 2011), in the regulation of a subset of SsrB-regulated virulence factors (Osborne and Coombes, 2009) and in the association with the Hfq-mediated regulation (Figueroa-Bossi et al., 2006).

1.4. Host immune system

Interactions of pathogens with mammalian cells are complex processes including numerous bacterial products, which are sensed by mammalian hosts. Myeloid-derived phagocytes, such as macrophages, monocytes, neutrophils, and dendritic cells are critical to control bacterial infections, and these cells have complementary functions to ensure host survival (Tam et al., 2008). Depending on cell types, a cascade of anti-bacterial processes is initiated by the initial binding and engulfment of pathogens. *Salmonella* can enter various types of the host cells by either SPI1-induced macropinocytosis or non-SPI1 phagocytosis depending on different cell types, and is initially enclosed in a spacious phagosomal compartment called the *Salmonella*-containing vacuole (Introduction 1.2.; Fig. 1) (Alpuche-Aranda et al., 1994). Depending on the mechanism of uptake, *e.g.* opsonization of the bacteria through complement proteins

(complement-mediated uptake), Fc-mediated uptake (binding of antibodies to the bacteria and binding of the Fc receptor), host cells are also activated to increase microbicidal and degradative as well as secretory functions (Webster et al., 2008). The initiation of the host innate immune response is also stimulated by the recognition of pathogen-associated molecular patterns (PAMPs). To date, the Toll-like receptor (TLR) family, which actives downstream signals, *e.g.* interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), are the most well-studied receptors for sensing PAMPs. Other classes of host receptors including NACHT [domain present in NAIP (neuronal apoptosis inhibitor protein), CIITA (class II transactivator), HET-E (plant het product), and TP-1 (telomerase-associated protein 1)]-leucine rich repeat receptors (NLRs) exist in the cell cytoplasm and activate intracellular PAMPs (Inohara et al., 2005).

1.4.1. Extracellular detection of pathogen-associated molecules

1.4.1.1. Toll-like receptors (TLRs)

Host receptors have developed a number of germline-encoded receptors to recognize PAMPs referred to as pattern-recognition receptors (PRRs) (Janeway and Medzhitov, 2002). PRRs in the innate immune system can be in a soluble, membrane-bound or cytosolic forms and function in both extracellular and intracellular recognition. One well-known family of PRRs is the Toll-like receptors (TLRs). TLRs contain several extracellular leucine-rich repeat (LRR) domains involved in direct or indirect pathogen recognition and an intracellular signal transducing domain called Toll/Interleukin-1 Receptor (TIR) domain. The TLR are transmembrane proteins localized at the cell surface or within phagosome/endosomes, thereby scanning the extracellular milieu for bacteria (Medzhitov, 2001). The Toll pathway was first discovered in *Drosophila* where it was found to function in the detection of both Gram-positive bacteria and fungi (Anderson et al., 1985a; Anderson et al., 1985b). Activation of TLRs leads to NF-κB-dependent production of innate immune cytokines such as TNF-α, IL-6 or IL-12, which contribute to the recruitment of inflammatory cells to the site during infection or inflammation.

1.4.2. Intracellular detection of pathogen-associated molecules

Recently two other families of signalling PRRs have been described as well: the Nod-like receptors (NLRs) and the RIG-like helicases (RLHs). 22 NLRs have been described in humans, among which are the nucleotide binding and oligomerization domain (NODs), NACHT-, LRR- and PYD-containing (Nalps) and neuronal apoptosis inhibitory protein

(Naip) (Fritz et al., 2006).

1.4.2.1. Nod1 and Nod2

Nod1 and Nod2 were the first two intracellular PRRs of the NLR superfamily to be identified (reviewed by Inohara and Nuñez, 2003; Le Bourhis et al., 2007; Murillo et al., 2003; Ogura et al., 2001). The NLR protein consists of an N-terminal effector domain followed by a central nucleoside-binding NACHT/NAD domain and a C-terminal LRR domain. Girardin et al. (2001) showed that invasive *Shigella flexneri* activates NF-κB in a Nod1-dependent manner after intracellular detection of the bacterium in epithelial cells and leads to production of pro-inflammatory mediators, such as IL-6 and TNF-α. Nod2 is another NLR protein, which is also able to activate NF-κB in a Receptor interacting protein-2 (RIP2)-dependent manner (Kobayashi et al., 2005).

Both Nod1 and Nod2 recognize components of peptidoglycan (PG) within the bacterial cell wall, although their specificities are different. Nod1 senses PG-derived meso-diaminopimelic acid (DAP)-containing muramyl peptides, which are structural componentes of the cell wall in Gram-negative bacteria (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003b). Magalhaes et al. (2005) reported that DAP-containing muramyl tripeptides could trigger Nod1 activity; however, murine Nod1 is also activated by DAP-containing muramyl tetrapeptides. Nod2, which is expressed in monocytes, macrophages, dendritic cells and intestinal epithelial cells senses muramyl depeptides (MDP) found in both Gram-positive and Gram-negative bacteria (Girardin et al., 2003c).

1.4.2.2. Nalps

Nalp proteins are characterized by C-terminal LRRs, a central NACHT/NAD domain and an N-terminal effector pyrin domain. In addition to Nalps, other pyrin domain-containing proteins have been found as well, *e.g.* the protein ASC (apoptosis-associated speck-like protein containing a CARD), which is composed of a C-terminal CARD and an N-terminal PYD (Chamaillard et al., 2003). Caspase recruitment domains (or called Caspase activation and recruitment domains [CARDs]) are interaction motifs found in numerous proteins, including helicases, kinases, mitochondrial proteins, caspases, and other cytoplasmic factors involved in the processes of inflammation and apoptosis (Hiscott et al., 2006; Hong and Junk, 2002). Pyrin domains (PYD domains or PAAD/DAPIN domain) are a subclass of protein motifs evolutionarily related to the Death domain family (Fairbrother et al., 2001; Pawłowski et al., 2001); it allows a pyrin domain containing a protein to interact with other proteins that

contain pyrin domains. Proteins containing a pyrin domain are frequently involved in biological processes called inflammation and apoptosis (Bertin and DiStefano, 2000). There are 14 Nalps, which are predicted to be located in the cytosol in human beings (Tschopp et al., 2003). Nalp2–14 all exhibit the following tripartite structure: PYD-NBS (non-binding site)-LRR. Interestingly, an additional CARD domain is found at the C-terminal end of Nalp1. Currently, Nalp1b has been shown to mediate caspase-1 activation and necrosis in response to the treatment together with anthrax lethal toxin (LeTx), which is composed of protective antigen and lethal factor (LF), within macrophages extracted from inbred mice (Boyden and Dietrich, 2006). Scobie and Young (2005) reported that lethal factor is able to translocate into the cytosol *via* protective antigen binds to the von Willebrand factor type A or integrin inserted domain containing an anthrax toxin receptor 1 and an anthrax toxin receptor 2, indicating that lethal factor might be the ligand directly or indirectly sensed by NALP1b. Liao and Mogridge (2009) also reported that Nalp1b recognizes activity of *B. anthracis* lethal toxin in the host cytosol to initiate anthrax lethal toxin-induced caspase-1-dependent cell death signaling pathways inside macrophages and dendritic cells. However, no evidence has been released that Nalp1b is able to recognize other types of toxins. Nalp3 (also known as cryopyrin or CIAS1) is recognized by multiple ligands (Masumoto et al., 2003). Similar to the NOD proteins, the human Nalp3 LRR is crucial for induction of IL-1 β secretion in response to muramyl dipeptide. Additionally, Nalp3 also acts as a sensor for non-microbial "danger" signals (Mariathasan, 2007). Frederick et al. (2008) reported that following neuronal injury is able to induce expression levels of death domain genes Nalp1 and Nalp5. Kempster et al. (2011) reported that the Nalp6 inflammasome and IL-18 function in mammalian intestines. Ohno et al. (2008) showed that the expression of Nalp7 is inducible in endometrial cancer tissues. Bovine Nalp8 and Nalp9 serve as oocyte marker genes (Dalbiès-Tran et al., 2005; Ponsuksili et al., 2006). Jéru et al. (2008) reported that a mutation of Nalp12 cause hereditary periodic fever syndromes. Westerveld et al. (2006) reported that Nalp14 has a function in spermatogenesis, which is the process of the cell division of male primary germ cells.

1.4.2.3. Ipaf, Naip

Ipaf and Naip are two members in the NLR family, which have been linked to the intracellular detection of bacteria. Both Ipaf and Naip bear C-terminal LRR domains and central NACHT domains; however, Ipaf contains a CARD in a N-terminal part and Naip presents 3 BIR domains in a N-terminal part (Fritz et al., 2006). *S.* Typhimurium (Lara-Tejero et al., 2006) and *Legionella pneumophila* (Amer et al., 2006) infections in macrophages result

in Ipaf-dependent activation of caspase-1, which leads to IL-1β maturation and caspase-1-dependent cell death. Ipaf has been implicated in the detection of flagellin using *S.* Typhimurium as an infection model system. Flagellin delivered to the cytosol activates caspase-1 through the Ipaf-mediated signal pathway. This process is independent of TLR5, which recognizes flagellin (Franchi et al., 2006). Naip5 also responds to flagellin in *S.* Typhimurium to initiate caspase-1-dependent macrophage cell death.

1.4.3. Host defense mechanisms

The first defense mechanism, which is triggered by invasive pathogens for phagocytic killing, is contributed by NADPH oxidase (Vazauez-Torres et al., 2000). Patients deficient in this enzymatic activity are susceptible to salmonellosis (Mouy et al., 1989). This mechanism includes the production of reactive oxygen species (ROS) and nitrogen molecules through phagocyte oxidase and inducible nitric oxide synthase, respectively. In addition to this oxygen-dependent microbicidal mechanism, engulfing of bacteria also stimulates host cells to release antimicrobial peptides (AMPs) into the lumen of the intestinal tract as one of the oxygen-independent mechanisms (Veldhuizen et al., 2009).

1.4.3.1. Host antimicrobial peptides (AMPs)

Host antimicrobial peptides form a crucial part of the human innate host defense for direct killing of a wide variety of bacteria. Most of these peptides are cationic in nature, and interact with the bacterial cytoplasmic membrane, which usually comprises negatively charged phospholipids (Eswarappa et al., 2008). These antimicrobial components, including lactoferrin (Lf), bactericidal/permeability-increasing protein (BPI), peptidoglycan recognition proteins (PRGP), serprocidins (NE, CatG, PR3 and Azu), neutrophil gelatinase-associated lipocalin (NGAL), histones, cathelicidins, lysozyme (Lz), phospholipase (PLA_2), calprotectin, granulysin and defensins perform different mechanisms to eliminate pathogens. In this study, I chose the subclasses of defensins and cathelicidin for further investigation.

1.4.3.1.1. Defensins

Defensins are small (M_r 3,500 – 4,000), cysteine-rich peptides comprising one of the major subclasses of AMP (reviewed by Ganz, 2003; Lehrer et al., 1989). They are classified into α-(HD) and β-defensins (hBD). Six α-defensins have been identified in human beings; two (HD-5 and HD-6) are expressed by Paneth-cells in the small intestine and in the female reproductive tract (Ouellette et al., 2001; Selsted and Ouellette, 2005). Among them, four

human neutrophil peptides (HNP)-1-4 are expressed mainly in neutrophils (Fahlgren et al., 2004). α-defensins express broad-spectrum microbicidal activity against bacteria, fungi, enveloped viruses, and parasites (Ayabe et al., 2000; Levy, 2004). The microbicidal activity of α-defensins is limited by the physiological concentrations of mono- and divalent cations as well as some components of plasma/serum (Levy, 2004). Another major subclass is the β-defensins, which are characterized by a distinct pairing of cysteines and have a higher lysine-to-arginine ratio than α-defensins (Lehrer et al., 2002). Based on the *in silico* analysis, 28 β-defensins have been predicted in the human genome (Schutte et al., 2002). Expression of human β-defensins is apparently restricted to epithelial cells including those of the respiratory tract (Ali et al., 2001; Singh et al., 1998). hBD-1 and -2 display antibacterial activity against Gram-negative bacteria (Valore et al., 1998), whereas hBD-3 is also active against Gram-positive bacteria and *Saccharomyces cerevisiae* (Garcia et al., 2001).

1.4.3.1.2. Cathelicidins

Cathelicidin is a putative cysteine-proteinase inhibitor, first isolated from pig leucocytes (Ritonja et al., 1989). The cathelicidin-protein family contains more than 20 members, which constitute a significant part of mammalian peptide antibiotics in a variety of species including humans (Agerberth et al., 1991; Agerberth et al., 1995; Storici and Zanetti, 1993). Most of the cathelicidin precursors are stored in cytoplasmic granules of neutrophil leucocytes. Upon leucocyte activation, mature forms of cathelicidins are released (Scocchi et al., 1992; Zanetti et al., 1991).

One member in the cathelicidin-protein family expressed in human beings is LL-37 (170 amino acids) (reviewed by Doss et al., 2010). LL-37 was isolated from human granulocytes and was detected in human wounds and blister fluids (Frohm et al., 1996). Recently, the gene encoding LL-37 was found to be induced in human keratinocytes during inflammatory disorders (Frohm et al., 1997). These findings suggest that LL-37 plays an important role in the first line of host defense against local infections and systemic invasion of pathogens at sites of inflammation and wound. LL-37 mediates a wide range of biological responses, including direct killing of microorganisms, chemotaxis and chemokine induction, regulation of inflammatory responses as well as adjuvant, angiogenic and wound healing effects (Nijnik et al., 2009). Although LL-37 is the first α-helical antimicrobial peptide isolated from a human source, it exhibits cytotoxic activity to eukaryotic cells as well, which is different from most known antimicrobial peptides (*e.g.* cecropin, magainin, dermaseptin) (Johansson et al., 1998). Recently, growing evidence indicates that the cathelicidin-protein

family have functions which extend beyond their antimicrobial activity (Gallo et al., 1994, Verbanac et al., 1993).

1.4.3.1.3. Thrombocidins

Thrombocidins are released from platelets and are derived from carboxy-terminal deletions of the CXC chemokines neutrophil-activating peptide II as well as connective tissue-activating peptide III in humans (Krijgsveld et al., 2000). Most of data regarding thrombocidin activity and the bacterial ability to resist thrombospondin are available for the rabbit peptides, which damage bacterial membranes. Thrombocidins are believed to be able to recognize other intracellular targets.

1.4.3.1.4. Polymyxin B

Polymyxin B, which is a cationic lipopeptide antibiotic derived from the bacteria *Bacillus polymyxia* is used to resist Gram-negative bacteria infection (Kwa et al., 2007; reviewed by Mitrophanov et al., 2008). It has a bactericidal action against almost all Gram-negative bacilli except the *Proteus* group (Falagas and Kasiakou, 2005; Russell, 1963). The basic structure of polymyxin B consists of a polycationic peptide ring and a tripeptide side chain with a fatty acid tail (Fig. 2) (Hancock, 1997). Polymyxin B is a mixture of at least four closely related components, polymyxin B_1 to B_4, with polymyxin B_1 and B_2 being the two major components (Kang et al., 2000; Orwa et al., 2001). The difference among these four components is only in the fatty acid moiety (Kang et al., 2000; Orwa et al., 2001).

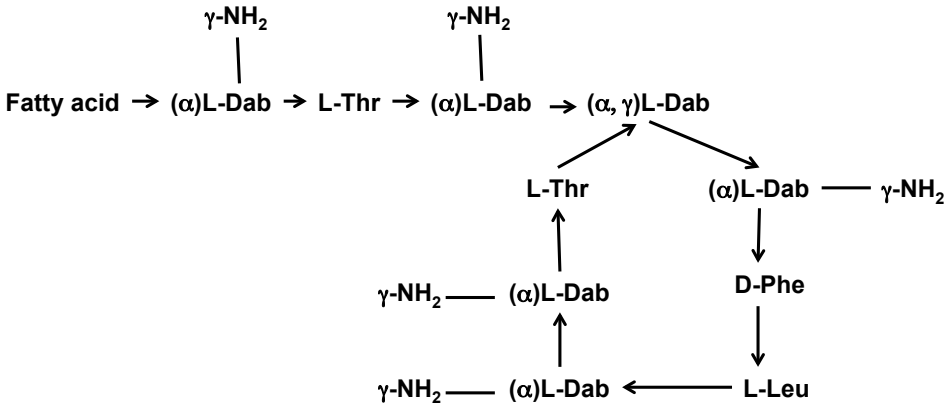


Fig. 2. The structure of polymyxin B. Fatty acid: 6-methyloctanoic acid for polymyxin B_1, 6-methylheptanoic acid for B_2, octanoic acid for B_3 and heptanoic acid for B_4. Thr, threonine; Leu, leucine; Dab, a,g-diaminobutyric acid; Phe, phenylalanine; where a and g indicate the respective amino group involved in the peptide linkage. The chemical structure of polymyxin B is drawn based on the reference published by Zavascki et al. (2007).

Polymyxin B is a rapid-acting bactericidal agent, with a detergent-like mechanism of action (Evans et al., 1999; Hermsen et al., 2003). The mechanism of polymyxins is to interact with bacterial LPS of Gram-negative bacteria and be subsequently taken up *via* the "self-promoted uptake" pathway (Hancock, 1997). The polycationic peptide ring therefore binds to the bacterial outer membrane and displaces the calcium and magnesium bridges, which are used to stabilize the structure of LPS (Evans et al., 1999; Hermsen et al., 2003). Fatty acid side chains of polymyxins further interact with LPS, contributing to the insertion of polymyxins into the outer membrane. Polymyxins are able to produce disruptive physicochemical effects, which lead to permeability changes in the outer membrane (Hermsen et al., 2003). The affected membrane is thought to develop transient breaks, which permit passage of various molecules, including hydrophobic compounds and small proteins and promote the uptake of the perturbing peptide itself and lead to cell death (Hermsen et al., 2003; Zavascki et al., 2007), hence the term "self-promoted uptake" (Hancock, 1997). In addition to its antibiotic function, polymyxin B has been used to clear endotoxin contamination in reagents (Suzuki and Shoji, 2010). It is also commonly used in the topical first-aid preparation Neosporin (Dasaraju et al., 1997).

1.4.3.2. Mechanisms of AMPs in elimination of intracellular pathogens

Membrane-targeting mechanisms are the most conserved killing mechanisms performed by AMPs. AMPs have been identified in many species, including plants, insects and vertebrate animals (Sang and Blecha, 2008). AMPs undergo a pore-forming process consisting of membrane attachment, insertion and permeabilization through positive charges on its surface as well as an amphipathic structure (Brogden, 2005). Currently, a two-state model, which is based on studies of representative cysteine-rich AMPs has been proposed. It is proposed that AMPs initially assemble parallel to the plane of the membrane and cause a membrane thinning in proportion to the peptide/lipid ratio of a membrane. Once peptides continuously assemble on membrane surfaces and exceed the peptide/lipid ratio threshold, the interaction is allowed to enter a second state, where the alignment of the peptide assemblages forms transmembrane pores (Huang, 2006; Jang et al., 2006).

1.5. Bacterial defense mechanisms against AMPs killing

Most AMPs function by forming multimeric pores in the cytoplasmic membrane of bacteria. As membrane integration by AMPs is dependent on the physicochemical properties of the membrane, mechanisms used by bacteria to resist AMP challenges can be achieved by

altering those properties, *e.g.* by altering the fluidity of the phospholipid bilayer through the introduction of fatty acids with different chain lengths (Jing et al., 2005; reviewed by Peschel, 2002). AMP resistance by bacteria includes two major mechanisms: efflux pumps and alteration of the bacterial cell surface composition.

1.5.1. Efflux pumps

Efflux is a general mechanism responsible for bacterial resistance to antibiotics. This active drug transport functions in a low intrinsic susceptibility, cross-resistance to chemically unrelated classes of molecules, and selection/acquisition of additional mechanisms of resistance to waste (Mahamoud et al., 2007). Multidrug resistance efflux pumps have been described in both Gram-positive and Gram-negative bacterial pathogens. For example, *Staphylococcus aureus* QacA-encoded energy-dependent efflux pumps are able to export toxic substances out of the cell (Rouch et al., 1990; Kupferwasser et al., 1999). Regarding Gram-negative bacteria, they are in general more resistant to antibiotics due to the sophisticated architecture of their cell membranes, including the outer and the inner membranes. The Gram-negative bacterial cell membrane contains a variety of protein channels used to transport (influx or efflux) various nutrition (sugars, amino acids, salts, metals) and noxious compounds (metabolites, drugs, biocides, detergents) (Li and Nikaido, 2004; Poole, 2005; Van Bambeke et al., 2003). These transporters are able to recognize toxic agents such as antibiotics and extrude agents from the periplasmic space to the extracellular environment, thereby reducing the intracellular accumulation of host bactericidals (Mahamoud et al., 2007). For example, the MtrCE system of *Neisseria gonorrhoeae* (Hagman et al., 1995), MexABOprK proteins of *Pseudomonas aenrginosa* (Poole et al., 1993) and AcrAE and EnvCD proteins of *Escherichia coli* (Pan and Spratt, 1994) belong to the group of bacterial efflux/transport proteins involved in resistance against drugs, dyes, and detergents.

1.5.2. Alteration of the bacterial cell surface composition

Prokaryotes produce a variety of glycoconjugates and polysaccharides, which contribute to the large structural diversities and complexities of the outer cell surface. These glycans include many unusual sugars, *e.g.* 3-deoxy-D-*manno*-octulosonic acid (Kdo), heptoses, and variously modified hexoses, which are important in the biology and bacterial pathogenicity (Horstman et al., 2004; Schnaitman and Klena, 1993).

In Gram-negative bacteria, the cell wall consists of inner and outer membranes separated by a space termed the periplasm. Peptidoglycan (PG) (also known as murein) makes

up approximately 10% of the dry weight of the cell wall and constitutes the major structural component of the periplasm. PG consists of parallel strands of polysaccharides composed of *N*-acetylglucosamine and *N*-acetylmuramic acid (MurNAc) in β1-4-linkage, which surround the bacterium (van Heijenoort, 2007). Each MurNAc is attached to a short (4- to 5-residues) amino acid chain, containing D-alanine, D-glutamic acid, and meso-diaminopimelic acid in Gram-negative bacteria and L-alanine, D-glutamine, L-lysine, and D-alanine in Gram-positive bacteria (Bouhss et al., 2008; van Heijenoort, 2007). The dibasic amino acid, *meso*-diaminopimelic acid (*m*-DAP) is responsible for the cross-link. The cross-linked structure of PG not only confers mechanical strength and shape on the cell wall, but also provides a barrier to withstand an internal osmotic pressure. Antibiotics, *e.g.* β-lactams and vancomycin, which inhibit PG biosynthesis, are the most widely used in current clinical practice (review by Koch, 2003). The largest family corresponding to β-lactams is an analogue of D-alanyl-D-alanine and mainly inhibits the transpeptidation, thus stopping cell growth (review by Gootz, 1990; review by Kitano and Tomasz, 1979). The most frequent mechanism used by bacteria against β-lactams is to produce β-lactamases to hydrolize the β-lactam ring (Bush and Jacoby, 2010; Wilke et al., 2005). Vancomycin is a glycopeptide, which binds to terminal D-alanyl-D-alanine and blocks the incorporation of peptides to the cell wall, thus inhibiting peptydoglicane elongation (review by Kahne et al., 2005). Bacteria are able to alter subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid, which recognized by vancomycin to decrease drug affinity (de Niederhäusen et al., 2011).

1.5.2.1. Modifications of teichoic acids and phospholipids

The permeability, thickness and physicochemical properties of the cell envelope are also involved in the susceptibility of bacteria against AMP challenges, since AMPs have to traverse the multi-layered bacterial cell envelope and reach the cytoplasmic membrane. For example, Gram-positive bacteria, *e.g.* Staphylococci, are remarkable because of its thick cell wall composed of peptidoglycan and teichoic acid polymers. Nannini et al. (2010) reported that vancomycin-resistance of *Staphylococcus aureus* (VRSA) is due to an increase in the cell wall thickness and remodelling of peptidoglycan in order to defend against AMP challenges.

1.5.2.2. Modifications of bacterial lipopolysaccharide (LPS)

1.5.2.2.1. Genetics of LPS biosynthesis

1.5.2.2.1.1. Lipid A

R-3-hydroxymyristoyl-acyl carrier protein (ACP), an unique early precursor of lipid A, is a

common intermediate, since it can be incorporated into the biosynthesis of lipid A and glycerol-based phospholipids (reviewed by Raetz and Whitfield, 2002; Raetz and Dowhan, 1990). The first committed step of lipid A biosynthesis is to transfer the acyl group from R-3-hydroxymyristoyl-ACP to the 3-OH group of UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is another central intermediate involved in cell surface biosynthesis and serves as the common precursor of peptidoglycan and lipid A. Acyl-transferase is encoded by *lpxA*, which belongs to a cluster of 11 genes organized into a complex operon termed the macromolecular synthesis II operon (Tomasiewicz, 1990). In addition to LpxA, this operon includes PolC, which encodes the α core subunit of DNA polymerase; the *rnh* gene, which encodes RNase H; and LpxB and LpxD, which are also involved in the lipid A biosynthetic pathway. Further steps involved in the lipid A biosynthesis pathway include N-deacetylation of the O-acylated from R-3-hydroxymyristoyl-ACP to the amino group in order to form UDP-2,3-diacylglucosamine referred to as a precursor of the non-reducing end of lipid A. LpxB functions to condense one molecule of UDP-2,3-diacylglucosame and one molecule of lipid X as well. This process leads to form a β-1,6-linked glucosamine disaccharide, in which each sugar is substituted with two 3-hydroxymyristoyl groups and carry a single phosphate residue on the first position of the reducing sugar (Crowell et al., 1987).

1.5.2.2.1.2. The LPS core region

The LPS core region always contains an oligosaccharide component that attaches directly to LPS lipid A and commonly contains sugars such as heptose and 3-deoxy-D-mannooctulosonic acid (also known as Kdo, keto-deoxyoctulosonate) (Hershberger and Binkley, 1968). LPS core regions in many bacteria also contain non-carbohydrate components, such as phosphate, amino acids, and ethanolamine substitutents. KdsA and KdsB are the first two gene products involved in biosynthesis of the LPS core region. The *kdsA* gene encodes the Kdo-8-phosphate synthetase, which combines D-arabinose 5-phosphate and phosphoenolpyruvate to generate Kdo-8-phosphate (Woisetschlager et al., 1987). Following the removal of the phosphate, free Kdo is activated to form CMP-Kdo by the CMP-Kdo synthase encoded by *kdsB* (Goldman et al., 1986). The third gene, *kdtA*, encodes a bifunctional enzyme (Belunis and Raetz, 1992), which is able to sequentially transfer two molecules from CMP-kdo to the lipid A precursor lipid IV_A. This process generates both Kdo backbone residues, Kdo I and Kdo II, which are linked in the α2,6- and the α2,4-position to the lipid A disaccharide, respectively. KdtA has been reported to serve as a transmembrane segment anchor (Clementz and Raetz, 1991). The late stage of the LPS core completion is to

add an additional α2,4-linked Kdo residue on Kdo II to form Kdo III (Schnaitman and Klena, 1993). KdtA has been considered to serve as a Kdo III transferase as well based on the study regarding the *Chlamydia trachomatis* GseA protein, which shares extensive regions of homology with the KdtA protein (Belunis et al., 1992). However, which gene encoding the hypothetical Kdo III transferase has not been identified yet (Schnaitman and Klena, 1993).

The *rfaD*, *rfaE*, *rfaC* and *rfaF* gene products function to synthesize heptose and the inner core. RfaE encodes ADP-heptose synthase (Sirisena et al., 1992); RfaD performs epimerization of ADP-heptose to L-glycero-D-manno form (Coleman, 1983); RfaC is involved in the addition of heptose I to Kdo I of the inner core region (Sirisena et al., 1992); and RfaF is involved in the addition of heptose II to heptose I in order to complete the inner core region backbone (Wilkinson et al., 1972). Furthermore, the function of the *rfaQGPSBIJYZK* operon is to sequentially attach glucose and galactose residues on the inner core region in order to provide the structural framework for the outer membrane and hexose region of the LPS core region (Carstenius et al., 1990; Creeger and Rothfield, 1979; Maclachian et al., 1991; Parker et al., 1992; Pradel et al., 1992). This operon is regulated by a positive regulator RfaH, which functions as a transcriptional antitermination factor (Pradel and Schnaitman, 1991).

1.5.2.2.1.3. LPS O-antigen

Functions of a second cluster of genes, the *rfb* cluster, comprising the *rfbBCAD*, *rfbKM* and *rfbFG* genes, are involved in the synthesis of TDP-rhamnose, GDP-mannose and CDP-4-keto-3,6-dideoxyglucose, respectively, and form the backbone of LPS O-antigen. In general, the backbone of LPS O-antigen consists of mannose, rhamnose and galactose and is identical in *Salmonella* serovars groups A, D and E1. The structure of *Salmonella* LPS O-antigen is represented in the following cartoon:

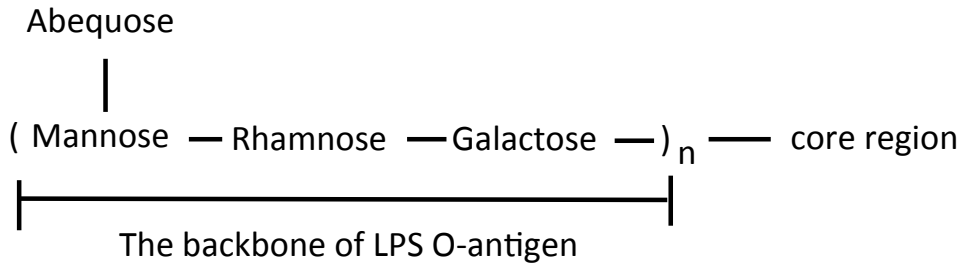


Fig. 3. *Salmonella* LPS O-antigen structure. The LPS O-antigen repeat unit contains mannose, rhamnose and galactose. Numerous LPS O-antigen repeat units form the backbone of *Salmonella* LPS O-antigen. Another sugar, abequose, is extended from mannose. n, numbers of LPS O-antigen repeat

nuits.

Mannose can be substituted for dideoxyhexose abequose and galactose can be partially replaced by glucose in other serotypes of *Salmonella* spp. Once sugars used for the synthesis of the LPS O-antigen backbone are ready to use, RfbP and Rfe function to initiate synthesis of LPS O-antigen repeat units. At present, the mechanism for the attachment of the first sugar on antigen carrier lipid (ACL) is divided into two classes, *rfbP*-dependent class and *rfe*-dependent class (Jann and Jann, 1984; Mäkelä and Stocker, 1984) in different genera of enteric bacteria. *Salmonella* spp, belong to the *rfbP*-dependent class further sub-divided into the O groups A, B, C2, D and E1. RfbP initiates synthesis of the LPS O-antigen repeat units by the addition of galactose-phosphate to ACL to form galactose-pyrophosphate-ACL; in contrast, Rfe initiates to synthesize of the O-antigen repeat units by the addition GlcNac-phosphate to ACL to form GlcNAc-pyrophosphate-ACL. The second stage for LPS O-antigen biosynthesis is to transfer those backbone sugars on either galactose-pyrophosphate-ACL or galactose-pyrophosphate-ACL. This process is controlled by a group of glycosyl transferases. For example, RfbN, RfbU and RfbV determine the rhamnose, mannose and abequose transferases, respectively. The rest of the genes, which are involved in O-antigen assembly and processing are *wzx*, *wzy* and *wzz*. The *wzx* gene encodes a flippase, which functions to transport LPS O-antigen repeat units across the inner membrane into the periplasmic space. The *wzy* gene encodes O-antigen polymerase, which functions to link each O-antigen repeat units *via* a glycosidic linkage and ligates them into a long LPS O-antigen chain. The *wzz* gene is involved in the determination of the LPS O-antigen chain length (Samuel and Reeves, 2003). *Salmonella* has been reported to contain two homologues of *wzz* genes. For details, see Discusstion section 5.3. (p. 91).

1.5.2.2.2. The Regulation of LPS modifications

1.5.2.2.2.1. Lipid A and the core region

Several enzymes have been reported to modify the fatty acyl chain region in LPS lipid A, including PagP, PagL, LpxR and LpxO. PagP, which is regulated by the PhoP/Q TCS, encodes a palmitoyl transferase located in the outer membrane; it transfers a palmitate from glycerophospholipids to the β2-position of LPS lipid A, resulting in a hepta-acylated structure (reviewed by Ernst et al., 2001; Ahn et al., 2004). Guo et al. (1998) also reported that this modification occurs during the period of LPS lipid A synthesis while LPS lipid A is transported to the inner leaflet of the outer membrane. The hepta-acylated structure of LPS

lipid A might prevent the insertion from host antimicrobial peptides (Bishop et al., 2008). *pagL* encodes 3-O-deacylase, which functions to remove the 3-O-linked acyl chain of LPS lipid A (Geurtsen et al., 2005); however, PagL plays no role in the resistance to AMP killing (Kawasaki et al., 2004). Although PagL is controlled by the PhoP/Q TCS, it is not active in the outer membrane while *Salmonella* was grown under the Mg^{2+}-limiting condition (Wang and Quinn, 2010). LpxR is another outer membrane protein, which removes the 3'-acyloxyacyl moiety from *Salmonella* LPS lipid A (Reynolds et al., 2006). LpxR is not regulated by neither the PhoP/Q- nor the PmrA/B TCS, but it requires the divalent cation Ca^{2+} for enzymatic activity (Rutten et al., 2009). Orthologs of *Salmonella* LpxR have been found in various Gram-negative bacteria including *Helicobacter pylori*, *Yersinia enterocolitica*, *E. coli* O157:H7 and *Vibrio cholera*. LpxO is an inner membrane protein, which generates a 2-OH group at the α,β-3'-position of *Salmonella* LPS lipid A (Gibbons et al., 2000; Gibbons et al., 2008). Like LpxR, LpxO is not regulated by the PhoP/Q- or the PmrA/B TCS.

The addition of amino groups on LPS lipid A is believed to be another important strategy for *Salmonella* to escape the host immune system. This function is mainly controlled by the PmrA/B TCS through the activation of genes whose products are involved in the addition of 4-amino-4-deoxy-L-arabinose (Ara4N) and phosphoethanolamine (pEtN). PmrE and PmrF contribute to add Ara4N on LPS lipid A (Gunn et al., 1998; Trent et al., 2001). This process occurs on the outer face of the inner membrane before LPS is flipped out to the outer membrane, where PmrC mediates the addition of pEtN to the 1-phosphate of LPS lipid A (Lee et al., 2004).

PmrG, which is encoded upstream of the *pmrHFIJKLM* operon, is involved in dephosphorylation of heptose II (HepII) in the LPS core region (Nishino et al., 2006). Other proteins, including LpxT, LmtA and LpxQ contribute to modify LPS lipid A as well. LpxT adds a second phosphate group at 1-phosphate of LPS lipid A by using undecaprenyl pyrophosphate as the substrate donor (Touzé et al., 2008). LmtA, which is a membrane enzyme in *Leptospira interrogans* transfers a methyl group from S-adenosylmethionine (SAM) to 1-phosphate of LPS lipid A (Boon Hinckley et al., 2005). In *Rhizobium*, LpxQ oxidizes glucosamine on LPS lipid A while O_2 is sufficient to form an aminogluconate unit (Que-Gewirth et al., 2003).

Taken together, these modifications increase positively charged moieties of LPS and affect the electrostatic interaction of cationic AMPs with the bacterial cell surface (Gunn et al., 1998; Zhou et al., 2001).

1.5.2.2.2.2. LPS O-antigen

The LPS O-antigen is an important component of the outer membrane of Gram-negative bacteria involved in resistance against host bactericidal compounds and which is localized at the outer surface of the bacterial membrane and protects bacteria from host killing (Samuel and Reeves, 2003). Various mechanisms have been found in numerous pathogens to modify or regulate structures and/or conformations of LPS O-antigen in order to avoid host challenges, *e.g. Escherichia coli* (Eder et al., 2009), *Salmonella* spp (reviewed by Gunn, 2008), *Helicobacter pylori* (Stead et al., 2005; Stead et al., 2010).

To date, several LPS-O-antigen modifications in many aspects of remodelling LPS O-antigen structures, acetylation and glucosylation modifications are found to increase structural diversities of LPS O-antigen (Whitfield et al., 2003). In *S.* Typhimurium, a PmrA/B-regulated gene, wzz_{ST} (*cld* or *rol*), which is located downstream of *pmrE* is involved in the determination of the LPS O-antigen chain length (Batchelor et al., 1991; Murray et al., 2003; Murray et al., 2006) (see section 5.3. for further details, page 91). Functions of acetylation and glucosylation occur after polymerisation of LPS O-antigen repeat units (Samuel and Reeves, 2003). The glycosyl transfer mediated by GtrB and GtrA takes place after LPS O-antigen repeat units have been translocated to the periplasmic face of the cytoplasmic membrane (Sanderson et al., 1995; Vander Byl and Kropinski, 2000). For instance, *S. enterica* serogroup B strains modify LPS O-antigen by the addition of glucose at the galactose residue (Vander Byl and Kropinski, 2000). This process occurs either *via* a (1→4) linkage, determined by chromosomal genes, or a (1→6) linkage, determined by the gene carried on the P22 lysogenic phage.

In addition to glycosylation, acetylation is a common form of LPS O-antigen modifications as well. Genes involved in LPS O-antigen acetylation could sometimes be found outside of the O-antigen cluster. For example, *Salmonella* OafA, which acetylates the second position of abequose locates outside of the main LPS O-antigen synthesis gene cluster. Slauch et al. (1995) reported that acetylation of O-antigen by OafA affects the three-dimensional structure of LPS. The function of OafA has been characterized. It belongs to a member of a family of integral membrane trans-acylases (Slauch et al., 1996) and determines the *Salmonella* O5-antigen (Slauch et al., 1995; Hauser et al., 2011). However, there are no further recent studies to investigate genetics of these phenomena in *Salmonella* strains. Kubler-Kielb et al. (2007) reported that bacteriophage-mediated glucosylation and acetylation of the LPS O-antigen specific chain are important for *Shigella flexneri* serotype 2a LPS antigenicity and consequently for immunogenicity of the LPS-based vaccines against

shigellosis. Additionally, increased variations of LPS O-antigen is also a strategy used by plant pathogens (Lerouge and Vanderleyden, 2002).

2. RATIONALE and AIM

In *S.* Typhimurium, the bacterial signal molecule, guanosine-3′,5′-bispyrophosphate (guanosine tetraphosphate or ppGpp), was found to play a pivotal role in the process of invasion and intracellular survival in *Salmonella* Typhimurium (Pizarro-Cerdá and Tedin, 2004; Thompson et al., 2006). ppGpp is a global regulatory molecule of gene expression in bacteria. Best known for its effects during amino acid starvation, ppGpp accumulates to high levels and causes a rapid down-regulation of stable RNA synthesis and ribosome production, a phenomenon known as the stringent response to amino acid deprivation. Mutants of *E. coli* were described which showed a "relaxed" response to amino acid deprivation, in which RNA continued to accumulate after amino acid starvation (Fiil and Friesen, 1968; Stent and Brenner, 1961). The gene was identified which was responsible for this effect and referred to as *rel* or *relA* (Alfoldi et al., 1962; Borek et al., 1956). Later, another gene involved in ppGpp metabolism was identified based on the absence of the spots on thin layer chromatograms of labelled guanosine nucleotides and referred to as *spoT*, in reference to the "spot-less" phenotype (Laffler and Gallant, 1974). SpoT was later found to be the major ppGpp-hydrolase responsible for degradation of ppGpp to allow recovery of RNA synthesis after amino acid starvations (Chaloner-Larsson and Yamazaki, 1976; deBoer et al., 1977; Fiil et al., 1977). It remained for many years unexplained why the low, basal levels of ppGpp were the same in *relA*+ (wildtype) and *relA* strains. Eventually, two, independent laboratories found that the basal levels of ppGpp present during normal growth in the absence of amino acid starvation were in fact synthesized by the SpoT gene product, which is also responsible for the degradation of ppGpp (Hernandez and Bremer, 1991; Xiao et al., 1991). The SpoT enzyme is responsible for adjustment of the basal ppGpp levels to match the availability of nutrients to the rate of ribosomal RNA (and ribosome) synthesis needed for maximal growth rates. The SpoT enzyme modulates the basal ppGpp levels in response to a number of different environmental/growth signals such as carbon source, metal ions, etc. Although the precise mechanism is not known, it is believed that the SpoT enzyme senses the charging status of transfer-RNAs (tRNAs). Current models therefore associate the RelA protein with the high level ppGpp synthesis during amino acid starvation conditions, the signal being uncharged tRNAs bound to the ribosome (RelA is ribosome-associated), whereas the growth-rate determining basal levels of ppGpp are synthesized by the cytosolic SpoT enzyme, either through low-level synthesis or adjustment of the rate of degradation.

A ppGpp-deficient (Δ *relA* Δ *spoT*) strain of *Salmonella* showed reduced expression of

nearly all SPI-encoded virulence genes of *S*. Typhimurium without affecting the expression of the major known global regulators (Thompson et al., 2006). The model proposed to explain this observation is based on the idea that in the absence of ppGpp, strong promoters such as the ribosomal RNA promoters and house-keeping genes which are generally GC-rich are capable of acquiring free RNA polymerase, whereas AT-rich promoters, including many of the horizontally-acquired virulence and pathogenicity-island associated genes, remain at a disadvantage based on the instability of such RNA polymerase: AT-rich promoter interactions.

In the previous study, a number of additional pathogenicity island- and virulence-associated genes were found to show severe reductions in expression by loss of Δ*relA*Δ*spoT*, as determined by microarray studies. Therefore, the working hypothesis in our laboratory has been that many of these uncharacterized genes may be involved in virulence and/or pathogenesis of *Salmonella*. The expression level of one putative transcriptional regulator STM0029 is strongly influenced by ppGpp (encoded by RelA and SpoT) in *S*. Typhimurium, implying that STM0029 might also be involved in *Salmonella* invasion and/or intracellular survival. Since there is no information or prior studies investigating the possible biological function of STM0029, I was therefore interested in the identification and characterization of the possible biological function(s) and involvement of STM0029 in virulence.

3. MATERIALS and METHODS

3.1. Molecular biology methods

3.1.1. DNA isolation

3.1.1.1. Plasmid preparation from *E. coli* and *Salmonella*

A single colony of bacteria was inoculated into 6 ml L-broth (Lennox, 1955) containing the appropriate antibiotic and incubated at 37ºC with shaking until the cultures reached OD_{600} approx. 2. Plasmid DNA of bacterial cultures was isolated by QIAGEN Plasmid Midi Kit (Qiagen, Inc.) according to the manufacturer's instructions.

3.1.1.2. Genomic DNA isolation

L-broth was inoculated with a signal colony of bacteria and incubated at 37ºC with shaking until the cultures reached OD_{600} approx. 2. The culture was centrifuged to pellet the bacteria and discarded the supernatant. High molecular weight chromosomal DNA was isolated by QIAGEN Genomic DNA preparation kit (Qiagen) according to the manufacturer's instructions.

To remove contaminating proteins and salts from DNA, the volume of the samples were adjusted to 300 µl with water and extracted with 500 µl of Tris-equilibrated phenol. The lower organic phase and the denatured proteins were discarded. The aqueous phase was further extracted twice with chloroform to remove traces of phenol and DNA was concentrated by ethanol precipitation.

One-tenth volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold 100% ethanol were added to the sample containing DNA. The mixture was mixed well by shaking and was allowed to cool at -80ºC for overnight. The sample was centrifuged at full speed for 1 hour next day. The pellet was washed twice with 70% ethanol, air-dried and re-suspended in sterile water.

3.1.2. RNA isolation

Total RNA was extracted from bacteria grown to the late log phage (OD_{600} approx. 2). To stabilise RNA, 5 ml of bacteria culture was added to 1 ml of 5% (v/v) phenol in ethanol maintained on ice followed by incubation for at least 30 minutes (Tedin and Blasi, 1996). The cells were centrifuged, resuspended in resuspension buffer (25 mM Tris-HCl, 1 mM EDTA) and lysed by adding an equal volume of 100ºC lysis solution (0.6 M NaOAc, 4 mM EDTA and 3% SDS) and boiling for 30 seconds to 1 minute. Repeated phenol and chloroform

extractions were performed to purify the lysate. Total RNA was treated with RNase-free DNase according to the manufacturer's instructions (Promega) to remove contaminating chromosomal DNA. The resultant RNA was further extracted with phenol and chloroform to obtain purified total RNA. DEPC-treated water was used in preparation of all the reagents.

3.1.2.1. Electrophoresis and detection of nucleic acids on agarose gels

Agarose gel electrophoresis was used for the routine analysis of DNA and RNA. Agarose gels were cast in 1X Tris-borate buffer (TBE) containing 0.5 μg/ml ethidium bromide. The DNA samples were mixed with DNA loading buffer and separated in agarose gels at 10 V/cm. The agarose concentration of the gels used was dependent upon the size of DNA fragments to be resolved, and ranged from 0.7 to 1.2% (w/v).

3.1.2.2. Determination of concentration of nucleic acids

The concentration of DNA and RNA were measured by Nanodrop Spectrophotometer (PeqLab) according to the manufacturer's instructions. Calculations were performed by used the absorption determined by the instrument and the following conversions:

1 unit of absorbance of dsDNA at a wavelength of 260 nm (A_{260}) = 50 μg/ml dsDNA
1 unit of absorbance of RNA a wavelength of 260 nm (A_{260}) = 40 μg/ml RNA

The A_{280} was also measured and the ratio between A_{260} and A_{280} was used to determine the purity of the sample. DNA sample are considered adequately pure if the ratio of A_{260} and A_{280} is between 1.6 to 1.8; RNA sample are considered adequately pure if the ratio of A_{260} and A_{280} is between 1.8 to 2.0.

3.1.3. Genome-wide microarray analysis

3.1.3.1. Nucleic acid extraction and purification

Bacterial culture samples (5 or 10 ml) were added to one-fifth of the sample volume of a solution of 5% (v/v) phenol in ethanol maintained on ice to stabilize total RNA. Total RNA was extracted and purified as described above. Chromosomal DNA was removed by digestion with 50-100 units of RNase-free DNase (Promega Corporation) and re-extracted with phenol and chloroform to obtain purified total RNA. The absence of contaminating DNA was verified by PCR involving primers targeting bacterial housekeeping genes.

3.1.3.2. Microarray construction

The 'Salsa' *Salmonella* serovar microarray was used in this study. Each 'Salsa' *Salmonella* serovar microarray consisted of 5080 protein coding regions or open reading frames (ORF), including 4414 *S*. Typhimurium LT2a (and pSLT) genes, 155 *S*. Typhimurium DT104 specific genes, 229 *S*. Typhimurium SL1344 specific genes, 196 *S*. Enteritidis PT4 specific genes and 86 *S*. Gallinarum 287/91 specific genes (http://www.ifr.ac.uk/Safety/Microarrays/default.html). The construction of the *S*. Typhimurium microarray has been described previously (Clements et al., 2002; Eriksson et al., 2003; Kelly et al., 2004).

3.1.3.3. Template labeling

RNA for microarray analysis was reverse transcribed into cDNA and labeled according to protocols described on the IFR microarray website (www.ifr.bbsrc.ac.uk/safety/microarrays/). Briefly, a random priming reaction consisting of 10 µg of total RNA and 5 µg of random hexamers (Invitrogen) was set up in a total volume of 9.4 µl of ultra-pure water (Sigma Aldrich) at 70ºC for 5 minute in the dark. Following incubation, the reaction was chilled on ice for 10 minutes and centrifuged. Finally, 4.6 ml of RT reaction mix, 2 µl of 1 mM Cy5-dCTP (GE Health Care Lifesciences) and 4 µl of Affinity script multi-temperature reverse transcriptase (Stratagene) were added to the random primed RNA, mixed and incubated at 25ºC for 10 minutes. The incubation was further continued overnight at 42ºC. The following day, RNA was hydrolysed by the addition of 15 µl of freshly prepared 0.1 M NaOH and the alkali was neutralized with a equal volume of 0.1 M HCl. Regarding to direct labeling of chromosomal DNA, 2 µg of *Salmonella* chromosomal DNA, brought up to 21 µl with ultra-pure water, 20 µl of 2.5X random primer/reaction buffer from BioPrime® DNA labeling system (Invitrogen) was added, boiled and chilled on ice for 5 minutes each. While on ice, components were added as followed: 5 µl of 10X dNTP mix, 3 µl of 1 mM Cy3-dCTP (GE Health Care Lifesciences) and 1 µl of Klenow enzyme. The reaction was centrifuged and incubated at 37ºC overnight in the dark. The labeled chromosomal DNA was used as a common reference and internal hybridization control in each experiment.

3.1.3.4. Hybridization

The hybridization was performed as indirect comparison (type II) experiments using genomic DNA as the common reference and internal hybridization efficiency control. Generally, the labeled cDNA and one-fifth volume of labeled genomic DNA (10 µl) were mixed and purified by using Qia-quick PCR purification kit (Qiagen) to remove

unincorporated/quenched Cy dyes. The labelled nucleic acid mixture was eluted twice with 50 µl ultra-pure water. To 10 µl of dried labeling reactions consisting of labelled RNA and genomic DNA, 5.625 µl of the hybridization buffer was added, incubated at 100ºC for 2 minutes and followed by a room temperature incubation for 5 to 10 minutes. The mixture was centrifuged twice at 13,500 rpm for 5 minutes and the supernatant was transferred to a new tube in order to remove particulate matter. The hybridization reaction was performed by pippetting the hybridization buffer to one edge for a coverslip placed on the genomic array placed in the hybridisation chamber [Custom made by Monterey Industries or purchased at Corning Costar (#2551)]. Humidity in the hybridization chamber was maintained by pippetting 20 µl of 3X SSC buffer on each side of the slide. The hybridisation chamber was place in a water bath at 63ºC overnight.

3.1.3.5. Washing and stringency for reduction of background

The array was washed twice directly placing the slides in a heated wash solution containing 2X SSC and 0.1% SDS at 63ºC and agitated for 5 minutes to remove the coverslips. A thermocouple attached to a hot-plate stirrer was used to maintain the temperature. The arrays were subsequently washed twice in 1X SSC and 0.2X SSC at room temperature for 5 minutes with agitation. The slides were further spin dried in an enclosed slide chamber at 12,000 rpm for 5 minutes at room temperature and stored in plastic slide chamber until scanning.

3.1.3.6. Data acquisition and analysis

The process for microarray data acquisition and analysis was based on instructions reported by Eriksson et al. (2003) and Kelly et al. (2004). Fluorescence intensities of scanned microarrays were quantified using GenePix Pro software, version 3.0 (Axon Instruments, Inc.). The data were filtered and spots showing a reference signal lower than background plus two standard deviations or obvious blemishes were omitted from evaluations. The local background was subtracted from spot signals, and fluorescence ratios were calculated. To compensate for unequal dye incorporation or any effects of the amount of template, data centering was performed by adjusting the median natural logarithm of the ratios for each group of spots printed by the same pin to zero. Only coding regions whose expression showed at least a two-fold difference (the median natural logarithm ≥ 0.33) in the experimental strain compared to the wildtype were further analysed. The significance of the data at $P = 0.05$ was determined using a parametric-based statistical test adjusting the individual P value with the Benjamini and Hochberg false discovery rate multiple test correction. All of the expression

data for genes discussed in the text have passed this filter and are therefore statistically significant. These tests are features of the Gene-SpringTM 6.2 (Silicon Genetics) microarray analysis software package, which was used for both data visualization and analysis.

3.1.4. Reverse-transcriptase PCR (RT-PCR) and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted and purified as described above. The RNA samples were further treated with RQ1 RNase-freeDNase (Promega, USA) at 37ºC for 4 hours and subsequently purified by phenol /chloroform extraction. The following day, purified RNA sample was utilised for further reverse-transcriptase reaction. A total of 2.0 µg of each RNA sample combined with 1 µg Random Primer (Promega, USA) was reacted at 70ºC for 2 minutes and subsequently stayed at 4ºC for following step. 5 µg M-MLV 5X Reaction Buffer (Promega, USA), 6.75 µl dNTPs (ROTI®-MIX PCR 1), 0.625 µl RNA inhibitor (Promega, USA) and 1 µl reverse transcriptase (Promega, USA) were added in each reaction and reacted at 37ºC for 1 hour. *dnaA* was used as internal controls for total RNA loaded on RT-PCR and qRT-PCR.

150 ng cDNA in 2 µl treated with 10.0 µl Power SYBR® Green Master Mix (Applied Biosystems, UK), 1.8 µl forward and reverse primers flanked an interested gene and 4.4 µl H_20 in total volume 20 µl was reacted in 96 well plate and follow the PCR program described in Supplement I (p. 132).

3.2. Microbiological methods

3.2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table I. The wildtype, virulent *S.* Typhimurium strain SL1344 (Hoiseth and Stocker, 1981) was originally obtained from B.A.D. Stocker and provided by F. Norel (Institut Pasteur) and re-isolated from the spleens of infected Balb/c mice. Bacteria were maintained in either L-broth (Lennox, 1955) or M9 minimal medium (Sigma-Aldrich). When necessary, the growth media were supplemented with antibiotics (Sigma-Aldrich, Carl Roth) at the following concentrations: kanamycin, 50 µg/ml; carbenicillin, 100 µg/ml; and chloramphenicol, 10 µg/ml. The cultures were plated on L-broth agar plates with appropriate antibiotics at 37ºC overnight and subcultured in L-broth liquid media (OD_{600} approx. 2) for following experiments; for strains containing the temperature-sensitive plasmid pCP20 or pKD46 that were growth at 30ºC. The medium was supplemented with carbenicillin and kanamycin for the selection of strains, respectively. M9 minimal medium was prepared by M9 Minimal Salts (5X) (Sigma-Aldrich), which contains 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 2.5 g/L NaCl and 5 g/L NH_4Cl.

Synthetic medium affecting SPI2 gene expression and SPI2-T3SS secretion have been described previously (Deiwick et al., 1999). The composition of PCN medium (TEKnova, Inc.) was as follows: 80 mM MOPS or 80 mM MES; 4 mM Tricine; 100 mM $FeCl_3$; 376 mM K_2SO_4; 50 mM NaCl; x (as specified in the Results section) mM K_2HPO_4/KH_2PO_4, 0.4 % glucose, 15 mM NH_4Cl; 1 mM $MgSO_4$, 10 mM $CaCl_2$; micronutrients (10 nM Na_2MoO_4; 10 nM $NaSeO_3$; 4 nM H_3BO_3; 300 nM $CoCl_2$; 100 nM CuSO4; 800 nM $MnCl_2$; 1 nM $ZnSO_4$).

3.2.2. Electroporation of bacteria

3.2.2.1. Preparation of competent bacteria

3.2.2.1.1. Preparation of electrocompetent bacteria for mutagenesis

Salmonella harbouring plasmid pKD46 was first inoculated into 6 ml L-broth containing 100 μg/ml carbenicillin at 30ºC for growth overnight. The pre-culture was sub-cultured into 500 ml L-broth with 1:100 dilution in following day at 30ºC. Once bacterial cultures reached an OD_{600} of approx. 0.6, expression of the the bacteriophage λ Red recombinase cloned under transcriptional control of the *araBAD* operon promoter on plasmid pKD46 was induced for 2 hours by the addition of arabinose in a final concentration of 5 mM for a further 2 hours. Bacterial culture was poured into 250 ml plastic tubes and centrifuged at 8,000 x *g* for 15 minutes at 4ºC. All subsequent manipulations were performed on ice. The pellet was resuspended in 250 ml with ice-cold 10% glycerol and re-centrifuged twice as above. The pellet was then carefully resuspended with 30 ml of 10% glycerol and centrifugated at 3,750 x *g* for 10 minutes and 1,650 x *g* for another 10 minutes. The supernatant was discarded and resuspended bacterial pellet in 2 ml of 10% glycerol. Aliquots of 100 μl were stored in pre-frozen 1.5 ml eppendorf tubes at -80ºC.

3.2.2.1.2. Preparation of electrocompetent bacteria for transformation with plasmids (rapid method)

A single colony of bacteria was used to inoculated into 6 ml of L-broth containing appropriate antibiotic and grown at either 30ºC or 37ºC depending on bacterial strains. Once bacterial culture reached OD_{600} approx. 2, 1 ml bacterial culture was transferred to 1.5 ml eppendorf tubes and centrifugated at 13,500 rpm 5 minutes at room temperature. Discard the supernatant and concentrate pellet of each 2 eppendorf tubes in 1 ml of ice-cold 10% glycerol. From this step onwards, all subsequent manipulations were performed on ice. Centrifugation was repeated with decreasing speeds of 6000 x *g*, 3000 x *g*, 2000 x *g* twice at 4ºC as follows.

Finally, the pellet was resuspended in 100 µl of 10% ice-cold glycerol in pre-frozen 1.5 ml eppendorf tubes and stored at -80ºC.

3.2.2.1.3. Electroporation

Electrocompetent bacterial cells were thawed on ice just before electroporation. One to two microliters of DNA preparation was added to the thawed competent cells and the mix was transferred to a 2 mm electroporation cuvette. EasyjecT Prima (PeqLab) apparatus was used for electroporation. The mix was pulsed at 2.5 kV according to standard protocols. The samples were immediately resuspended in 1 ml L-broth without antibiotics and incubated for one hour at room temperature. Transformants were selected by plating onto selective agar plates followed by incubation at 32°C or 37°C overnight.

3.2.3. Bacteriophage

3.2.3.1. Preparation of P22 phage stocks

Bacteria were inoculated in 6 ml L-broth containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$ and grown to OD_{600} approx. 2 at 37ºC. 5 to 10 µl of bacterial culture was transferred to a pre-warmed sterile test tube containing 6 ml of 0.7% soft agar, 10 mM $MgSO_4$ and 5 mM $CaCl_2$ and maintained at 70ºC before adding bacterial culture. The mixture of warm soft agar and bacteria was immediately poured onto a LB agar plate and left for 10 minutes at room temperature to allow hardening of the soft agar. 100 µl of a dilution of the wildtype P22 particles were spotted onto the soft agar and spread by tilting the plates. The dried plates were incubated overnight at 37ºC. The following day, cleared zones on the soft agar plates were collected using a sterile spatula and transferred to a 15 ml polypropylene tube (Falcon). 1 ml L-broth containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$ and 1 µl of chloroform were added in to polypropylene tube and incubated overnight at 4ºC to allow diffusion of the bacteriophage out of the agar. The following day, the tubes were centrifuged at 7,500 rpm 15 minutes at 4ºC to remove the agar and cell debris. The clear supernatants were transferred to eppendorf tubes and stored at 4ºC for further usage. 10 µl of chloroform/ml supernatant was added into each phage stock to prevent bacterial growth and maintain sterile stocks of bacteriophage.

3.2.3.2. Transduction of *Salmonella* with phage P22

The recipient bacterial strain was cultured to OD_{600} approx. 2 in 6 ml L-broth containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$ at 37ºC. 2 to 5 µl of the donor P22 phage lysate was further added to 100 µl of the bacterial culture and incubated at 37ºC for 20 minutes. EGTA was

added with a final concentration of 20 mM to chelate the metal ions necessary for phage infection to stop the reaction. The volume of the mixture was brought to 1 ml with L-broth containing 20 mM EGTA and 100 µl aliquots of the mixture was plated on L-broth agar plate containing the appropriate antibiotic. The resulting transductants were further screened on green plates (Sternberg and Maurer, 1991). For the protocol of standard green plates see Supplementary Table I (p. 132). to eliminate lysogens and infected cells. Infected bacterial colonies are dark blue/green due to lysis of bacterial cells followed by a pH change, whereas uninfected colonies maintain a pale green colour.

3.2.3.3. Generation of mutants

All experimental strains constructed in this study are isogenic derivates of the wildtype *S.* Typhimurium SL1344 strain which have the indicated deleted genes replaced by a kanamycin resistance cassette using the bacteriophage recombinase (Datsenko and Wanner, 2000; Murphy, 1998; Zhang et al., 1998). For strains where no kanamycin cassette is indicated at the site of deletion, the kanamycin cassette has been removed by introduction of the plasmid pCP20, encoding the yeast FLP resolvase, followed by loss of the plasmid by growth at 37°C (Cherepanov and Wackernagel, 1995). Single-copy *lacZ* transcriptional fusion strains in various strain backgrounds were constructed by using plasmid pKG136 as described for a related plasmid (pCE36) (Ellermeier et al., 2002).

3.2.3.4. Polymerase chain reaction (PCR)

The DNA fragment was amplified by the standard BioTherm Taq polymerase (Rapidozym). For the protocol of standard PCR reactions see Supplementary Table I (p. 132).

3.3. Biochemical methods

3.3.1. β-galactosidase assay

Expression of *lacZ* fusions was assessed using β-galactosidase assays as described by Thompson et al., (2006). Bacterial cultures were growth to the late log/early stationary phase of growth (OD_{600} approx. 2) in L-broth or subcultured 4 hours in the M9 minimal medium. 1 ml culture was transferred to reaction tubes containing 1 ml Z buffer. 25 µl 0.1% SDS and 50 µl chloroform were added to each tube to permeabilized the bacteria. Samples of 0.4 ml (4 mg/ml) o-Nitrophenyl- β -D-galactopyranosid (ONPG) (Sigmal-Aldrich) were added to start the reaction, and 1 ml of 1M $NaCO_3$ was added to stop the assay. Samples were determined of absorbance at 420 nm and culture density was assessed at 550 nm.

3.3.2. In vitro assays for assessing tolerance to antimicrobial peptides (AMPs)

The wildtype *S.* Typhimurium SL1344 and each mutant strains were propagated in L-broth until OD_{600} approx. 2 and the assay was followed the protocol described by Fields et al. (1989) with slightly modifications. Bacteria (10^5 cfu pro well) were seeded into a 96-well plate (Costar) containing one of the substances diluted in appropriate media with indicated concentrations and incubated at 37°C for 1 hour. Polymyxin B (Biochrom AG) was reconstituted in the 0.1% sodium chloride buffer; human α-defensin-1, β-defensin-2 and LL-37 (Peptide Institute, INC., Japan) were diluted by the Iscove's modified Dulbecco's Eagle's medium as the minimal medium. Samples from each concentration were plated at different dilutions in LB plates. For the β-defensin-1 reaction, 10 mM potassium phosphate buffer was used since sodium chloride is known to inhibit the activity of β-defensin-1 (Singh et al., 1998). Data are presented as percentage survival relevant to the untreated sample.

3.3.3. Serum sensitivity assay

To analyse the sensitivity of various *Salmonella* strains to serum complement, bacteria were grown in L-broth, washed twice in phosphate-buffered saline containing 5 mM $MgCl_2$ (PBSM), and diluted in PBSM to 1×10^8 cfu/ml. To test serum sensitivity, 50 μl of the bacterial suspension was mixed with an equal volume of 100% normal non-heated sera (NNS) or 100% heat-inactivated sera (HIS) and incubated for 60 minutes at 37°C, respectively. Serial dilutions of the samples were prepared in PBSM and plated onto MH plates to determine the number of viable bacteria. The percent survival of the wildtype strain was calculated as follows: 100 × [mean (cfu ml^{-1} in NNS/cfu ml^{-1} in HIS) of mutant]/[mean (cfu ml^{-1} in NNS/cfu ml^{-1} in HIS) of wildtype].

3.4. Cell culture methods

3.4.1. Cell culture preparation

J774A.1 (ATCC TIB-67) murine macrophage cells were used in this study for cell culture and cell invasion assay. Briefly, cells were grown and maintained in Iscove's modified Dulbecco's Eagle's medium (Biochrom AG). The medium was supplemented with 10% fetal calf serum (PAN Biotech) at 37°C and 5% CO_2. To passage culture cells, the cells were first washed with 1X PBS and treated with Trypsin/EDTA solution at 37ºC for 5 minutes to loosen

the attachment of the cells. The cells were centrifuged and washed with appropriate medium to remove trypsin and an aliquot of the cell culture was added in the cell culture flask.

3.4.2. Preparation of stocks

The cells were suspended in appropriate culture medium to obtain the concentration of 10^7 cell/ml. Sterile dimethyl sulfoxide (DMSO) was added to a final concentration of 10% and frozen overnight at -80ºC. The stocks were transferred to liquid N_2 for long term storage on next day.

3.4.3. Cell invasion assay

The bacteria were grown with aeration to an OD_{600} of between 2 and 3 (late log phase), collected by centrifugation, resuspended in cell culture medium, and diluted to the appropriate concentrations for infection. Duplicate wells of monolayers of cells grown in 24-well culture plates (10^5 cells per well) were infected to yield a multiplicity of infection of ≤ 1 per host cell. After a centrifugation step (5 min, 250 × *g*), infected cells were incubated for 30 minutes prior to a change of medium containing 50 µg/ml of gentamicin (Biochrom AG). The medium was again replaced after 60 minutes with medium containing 10 µg/ml of gentamicin for the remainder of the experiment. At the times indicated in the figures, the wells were washed twice with phosphate-buffered saline and lysed by addition of 0.1% Triton X-100 in distilled water. Serial dilutions of lysates were plated for determination of intracellular bacteria. The data shown are representative of at least three independent assays for all strains.

Table I. List of bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
Strains		
KT2958	SL1344 wildtype *S.* Typhimurium hisG46 rpsL	Hoiseth and Stocker, 1981
KT5476	SL1344 hisG46 rpsL Δ STM0029::kan	This study
KT6192	SL1344 hisG46 rpsL Φ (PSTM0029::*lacZY*::kan)	This study
KT6266	SL1344 hisG46 rpsL Φ (P*pagD*::*lacZY*::kan)	This study
KT6268	SL1344 hisG46 rpsL Φ (P*pagP*::*lacZY*::kan)	This study
KT6272	SL1344 hisG46 rpsL Φ (P*pagD*::*lacZY*::kan) Δ STM0029	This study
KT6274	SL1344 hisG46 rpsL Φ (P*pagP*::*lacZY*::kan) Δ STM0029	This study
KT6342	SL1344 hisG46 rpsL Δ *pmrAB*::kan	This study
KT6360	SL1344 hisG46 rpsL Φ (P*pagC*::*lacZY*::kan)	This study
KT6362	SL1344 hisG46 rpsL Φ (P*pagC*::*lacZY*::kan) *phoP*60::Tn10d(Tc)	This study
KT6364	SL1344 hisG46 rpsL Φ (P*pagD*::*lacZY*::kan) *phoP*60::Tn10d(Tc)	This study
KT6366	SL1344 hisG46 rpsL Φ (P*pagP*::*lacZY*::kan) *phoP*60::Tn10d(Tc)	This study
KT6478	SL1344 hisG46 rpsL Φ (P*pagC*::*lacZY*::kan) Δ STM0029	This study
KT6480	SL1344 hisG46 rpsL Δ *pmrAB* Φ (P*pagC*::*lacZY*::kan)	This study
KT6482	SL1344 hisG46 rpsL Δ *pmrAB* Φ (P*pagD*::*lacZY*::kan)	This study
KT6484	SL1344 hisG46 rpsL Δ *pmrAB* Φ (P*pagP*::*lacZY*::kan)	This study
KT6486	SL1344 hisG46 rpsL Δ *pmrAB* Φ (PSTM0029::*lacZY*::kan)	This study
KT6488	SL1344 hisG46 rpsL Φ (PSTM0029::*lacZY*::kan) *phoP*60::Tn10d(Tc)	This study
KT6546	SL1344 hisG46 rpsL Φ (P*pcgL*::*lacZY*::kan)	This study
KT6548	SL1344 hisG46 rpsL Φ (P*pcgL*::*lacZY*::kan) Δ STM0029	This study
KT6778	SL1344 hisG46 rpsL Δ *pmrAB* Φ (P*pcgL*::*lacZY*::kan)	This study
KT6782	SL1344 hisG46 rpsL Φ (P*pcgL*::*lacZY*::kan) *phoP*60::Tn10d(Tc)	This study
KT7074	SL1344 hisG46 rpsL Δ*oafA*::kan	This study
Plasmids		
pCP20	*bla cat* pSC101(ts) cI857(ts) pL-FLP+	Cherepanov and Wackernagel, 1995
pKD4	*bla kan* oriRg (pir-dep.)	Datsenko and Wanner, 2000
pKD46	*bla araC* pBAD-g-b-exo repA101(ts) oriR101	Datsenko and Wanner, 2000
pKG136	*kan*(*aph*) FRT *lacZY*+ oriR6K	Ellermeier et al., 2002

Table II. List of primers used in this study

Primer Name	Sequence
dnaAFor	GGCGAAGTAGCGTTCTTTAT
dnaARev	TTCACGCAGTTGCTCAATTT
k1	CAGTCATAGCCGAATAGCCT[a]
k2	CGGTGCCCTCAATGAACTGC[a]
Km	CGATCGCTGTTAAAAGGACA[a]
Lac	GACCATTTTCAATCCGCA[b]
oafAFor	AAACATTATGATATTGAGTT
oafARev	GCTTGCAGTTCTCTGCGTAA
P1	TGTGTAGGCTGGAGCTGCTTCGA
P2	CATATGAATATCCTCCTTAG
rfaHFor	GCTCTTTCCTAATTATCTGT
rfaHRev	TAAGCAGTAACATCGAACGC
rfeFor	GTAGTACACTGATTGGCTTT
rfeRev	ATGAGAGTATTCTGCCGTTA
STM0029H1P1	GAAACGTACCTTATGAATATCAAAACGGAGTGAATTC**ATG**TGTGTAGGCTGGAGCTGCTTCGA[c,d]
STM0029H2P2	GTTCTCACCTTCCCGGGACACGGTCTACAGACTTTATCT**TCA**CATATGAATATCCTCCTTAG[e,f]

[a] Primers are designed according to Datsenko and Wanner (2000)

[b] Primers are designed according to Ellermeier et al. (2002)

[c] Bold letters represent the start code of the STM0029 gene

[d] Underlined bases represent the P1 primer sequence according to Datsenko and Wanner (2000)

[e] Bold letters represent the stop code of the STM0029 gene

[f] Underlined bases represents the P2 primer sequence according to Datsenko and Wanner (2000)

4. RESULTS

4.1. *Salmonella* STM0029 contributes to the resistance to antimicrobial peptide killing

In a previous study, a ppGpp-deficient strain of *Salmonella* (Δ *relA* Δ *spoT*), which is non-invasive and avirulent in mice, showed severe reductions in expression of nearly all genes previously shown to be involved in invasion and intracellular survival and replication (Thompson et al., 2006). In addition, many previously uncharacterized genes and open reading frames (ORFs) also showed altered patterns of expression. Based on the hypothesis that many of these genes might be involved in virulence or pathogenesis (see Introduction), I set out to determine in this study the possible role(s) of one of these candidate genes, a putative transcriptional regulator referred to as STM0029. STM0029 (NP_459034) is an open reading frame, which consists of 447 base pairs. The STM0029 gene is flanked by STM0028.1n, which encodes a hypothetical protein and STM0030, which is predicted to encode a putative transcriptional regulator belonging to the LysR family (Fig. 4A).

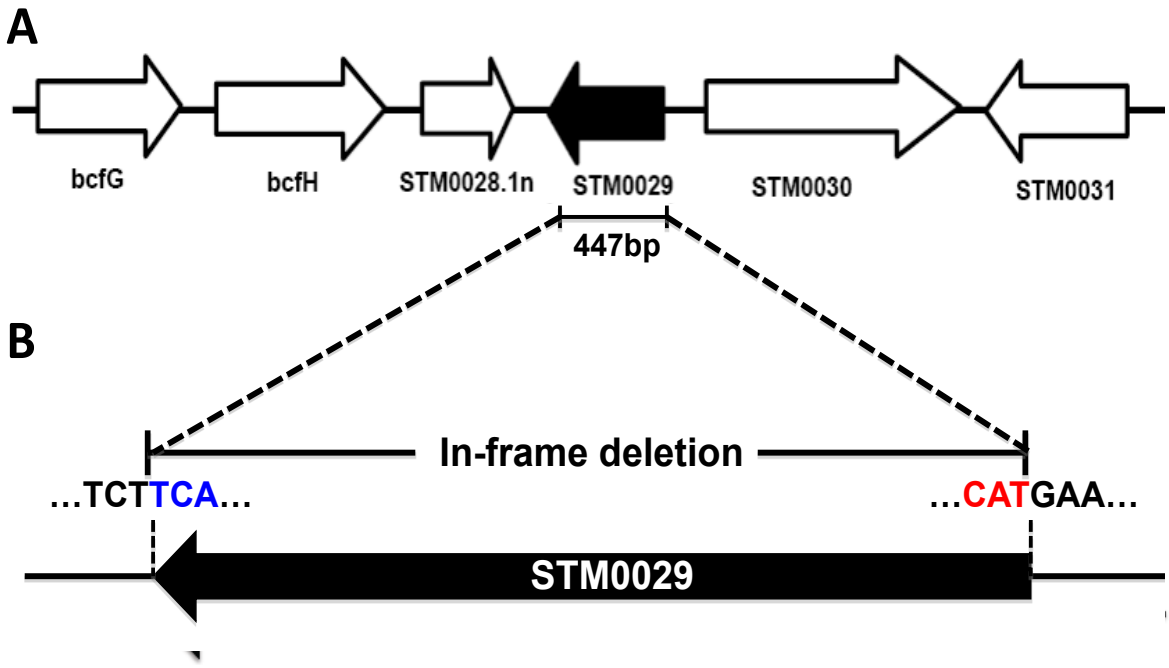


Fig. 4. The organization of the *Salmonella* genome around STM0029. (A) The cartoon shows the gene organization of the chromosomal region around the STM0029 open reading frame at bp 2440125-2441063 of the *Salmonella* genome. STM0029 is predicted to encode a putative transcriptional regulator of 129 amino acids. (B) The position of the primers (STM0029H1P1 and STM0029H2P2) used to carry out completed in-frame deletion of the STM0029 gene. Red letters represent the start codon of STM0029; blue letters represent the stop code of STM0029.

First, a directed, chromosomal deletion mutation of STM0029 (Fig. 4B, p. 53) was constructed via λ Red recombinase mutagenesis, verified by PCR and transferred back into the isogenic wildtype strain *via* bacteriophage P22 transduction (see section 3.2.3.2., p. 47). Colony morphology and growth kinetics of both the wildtype and mutant strains in culture showed no significant differences, confirming the mutation did not confer a general growth defect (data not shown).

Initially in this study, host cell invasion assays were performed with a murine macrophage cell line (J774A.1) during different independent time periods (2008 - 2010 and 2011) with bacterial strains including the wildtype, ΔSTM0029-, Δ*pmrA/B*-, and Δ*oafA* mutants because host cells (both phagocytes and non-phagocytic cells) have been known to employ multiple mechanisms to kill intracellular pathogens, including the release of pore-forming cationic antimicrobial peptides (AMPs). Briefly, equal amounts of the wildtype and mutants (MOI=1) grown to an OD_{600} between 2 and 3 (late log phase) were used to infect macrophages (see section 3.4.3. for details, p. 50). Samples were collected after 2, 4 and 24 hours post-infection. Based on results obtained in 2008 – 2010, the ΔSTM0029 and Δ*oafA* strains showed similar rates of host cell invasion to the wildtype after 2 and 4 hours post-infection, respectively; however, the intracellular bacteria (cfu) present at time points post-infection for both mutant strains showed about 50% reductions compared to the wildtype 24 hours post-infection (Table III, see page 56 below). In contrast, the Δ*pmrA/B* strain exhibited both reduced invasion and intracellular survival at both early time points (2- and 4 hours post-infection) and the late time point (24 hours post-infection). Based on the "doubling time" (g) (open source offered by Dr Alan Cann) (http://www.microbiologybytes.com/LabWork/bact/bact18.htm), the mean intracellular generation time was calculated after bacteria infected macrophages. The formula used for calculation is as follow:

$$N_g = (\log_{10} N_t - \log_{10} N_0) / \log_{10} 2$$

$$g = (T_t - T_0) / N_g$$

N_g : the number of generations

N_0 : the number of bacteria at the zero time point

N_t : the number of bacteria at the t time point

g : doubling time or mean generation time

As shown in Table III, the mean intracellular generation time obtained from the ΔSTM0029, the Δ*pmrA/B* and Δ*oafA* strains showed reduced growth from 2 to 24 hours post-infection compared to the wildtpye (data from 2008 - 2010). However, when these same experiments were repeated in 2011, the intracellular survival rates obtained for the ΔSTM0029 and Δ*pmrA/B* strains were not as severely reduced as previously observed compared to the wildtype 24 hours post-infection. Furthermore, the intracellular cfu for the Δ*pmrA/B* strain were much higher at the early time points 2 and 4 hours post-infection as observed in 2008 – 2010. Unexpectedly, the Δ*oafA* strain had lost its original phenotype in macrophages. At this time we have been unable to explain the discrepencies between these independent experiments, possibly a change in the source or charge of serum used in the cell culture medium may be responsible (see Discussion 5.1. for further details, p. 88). Although the phenotypes of the ΔSTM0029 and the Δ*oafA* strains were therefore not consistently reproducible in the macrophage cell lines in independent experiments, the ΔSTM0029 strain appeared to show a macrophage-specific defect for intracellular survival in macrophage cell lines compared to the wildtype. A macrophage-specific defect in intraceulluar survival is a rare phenotype *Salmonella*, which implied a role for STM0029 in *Salmonella* intracellular survival and/or pathogenesis. Such phenotypes have been reported in *Salmonella* strains with mutations of SPI2 effectors, including SsaR, SifA (Brumell et al., 2001), SpiA (Ochman et al., 1996), SsaV, SsaM (Yu et al., 2004) and the PhoP/Q TCS (Fields et al., 1986).

Table III. Phenotypic characterization of bacteria after infecting macrophages[a]

	Bacterial strains			
	Wildtype	**ΔSTM0029**	**Δ*pmrA/B***	**Δ*oafA***
Experiments performed in 2008 - 2010				
Time after infection (h)	**Intracellular cfu (% input)[b]**			
2	40.11	36.42	6.74	54.77
4	70.85	54.40	15.72	66.83
24	613.61	213.24	35.56	349.74
Time intervel after infection (h)	**Mean intracellular generation time (g)[c]**			
2 - 24	5.24	8.06	9.17	8.22
Experiments performed in 2011				
Time after infection (h)	**Intracellular cfu (% input)[b]**			
2	56.68	43.33	31.52	56.60
4	78.27	54.65	61.21	60.75
24	1067.11	575.32	620.2	975.93
Time intervel after infection (h)	**Mean intracellular generation time (g)[c]**			
2 - 24	5.19	5.89	5.11	5.35

[a] Murine macrophage cell line J774A.1 was used for cell invasion assays.

[b] The intracellular survival rate was calculated with a formula:

$100 \times$ [mean (cfu ml^{-1}at the indicated time)]/[mean (cfu ml^{-1} from input)]

[c] Mean intracellular generation time (g) was calculated with a formula described in the text. The value of mean intracellular generation time (g) indicates the doubling time in hours.

Phagocytes in particular are known to produce a number of AMPs involved in elimination of intracellular bacteria (Sang and Blecha, 2008; Wiesner and Vilcinskas, 2010). To address this question, I therefore investigated the suseptibility of the ΔSTM0029 strain after the treatment of human α-defensin-1, β-defensin-1 and β-defensin-2 (Fig. 5, p. 58; also see section 1.4.3.1., p. 27). The susceptibility of strains with a mutant in *phoP* and *pmrA/B* was also investigated in this study, respectively, since one of the major functions contributed by the PhoP/Q- and the PmrA/B TCS has been reported to be involved in the resistance to AMP killing (Fields et al., 1989; Mäkelä et al., 1978; Miller et al., 1990; Roland et al., 1993; Shi et al., 2004). The ΔSTM0029 strain showed similar susceptibility patterns as the Δ*pmrA*/*B* strain against α-defensin-1 (Fig. 5A, C) and β-defensin-2 (Fig. 5G, I), although the Δ*pmrA*/*B* strain appeared somewhat tolerant to β-defensin-1 compared to the wildtype, with bacterial killing apparent only at the highest concentration (Fig. 5F). The Δ*phoP* strain showed susceptibilities against α-defensin-1 (Fig. 5B) and β-defensin-2 (Fig. 5H); however, in contrast to the ΔSTM0029 and the Δ*pmrA*/*B* strain, the Δ*phoP* strain was apparently tolerant to β-defensin-1 (Fig. 5E).

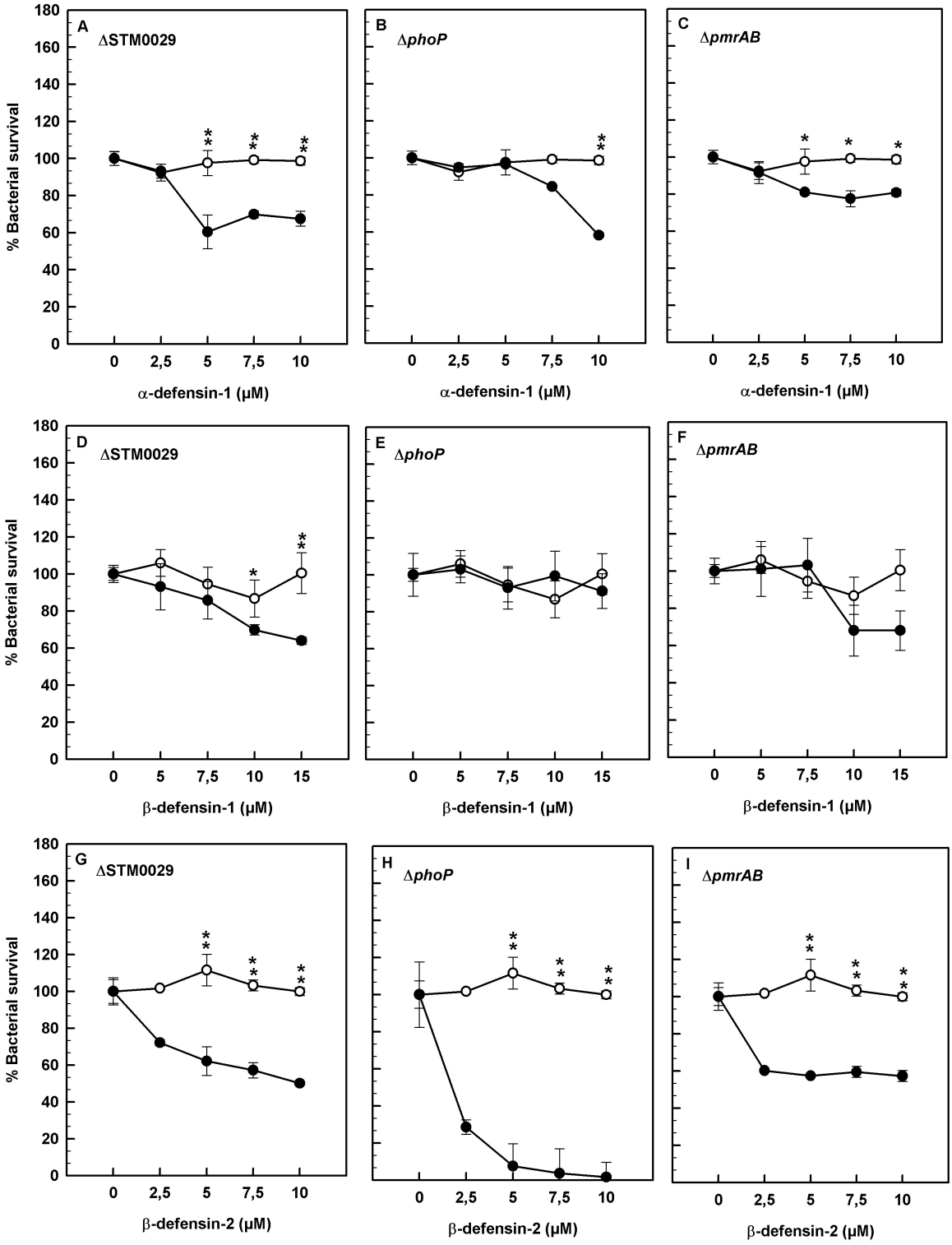


Fig. 5. The ΔSTM0029 strain is sensitive to human defensin killing. The wildtype (○, open circles) and mutant strains (●, filled circles) were grown in L-broth to an OD_{600} of approx. 2 and were treated with the indicated concentrations of defensins, including α-defensin-1 (A, B, C), β-defensin-1 (D, E, F), and β-defensin-2 (G, H, I), respectively. Bacteria were incubated with each AMP at 37°C for 1 hour and dilutions of the bacterial samples were plated for determination of colony-forming units (cfu). Percent survival is the mean ± standard deviation (SD) of three independent experiments with

similar results. All experiments were performed in triplicate measurements, and error bars indicate standard deviations. *, $P < 0.01$; **, $P < 0.005$.

In addition to defensins, the cathelicidin-protein family is another main group of host AMPs (Nijnik et al., 2009; Wu et al., 2010; also see section 1.4.3.1., p. 27). Cathelicidin expressed in humans is called LL-37 (170 amino acids). LL-37 is expressed in various cell types, including both phagocytic leucocytes and epithelial cells. As shown in the figure 6, susceptibility of the ΔSTM0029 strain (Fig. 6A) to LL-37 was similar to that found in the Δ*pmrA*/*B* strain (Fig. 6C); however, the killing pattern obtain from the ΔSTM0029 strain was intermediate between the highly sensitive Δ*phoP* strain (Fig. 6B) and the Δ*pmrA*/*B* strain. These observations indicated that STM0029 is involved in *Salmonella* resistance against mammalian AMP challenges.

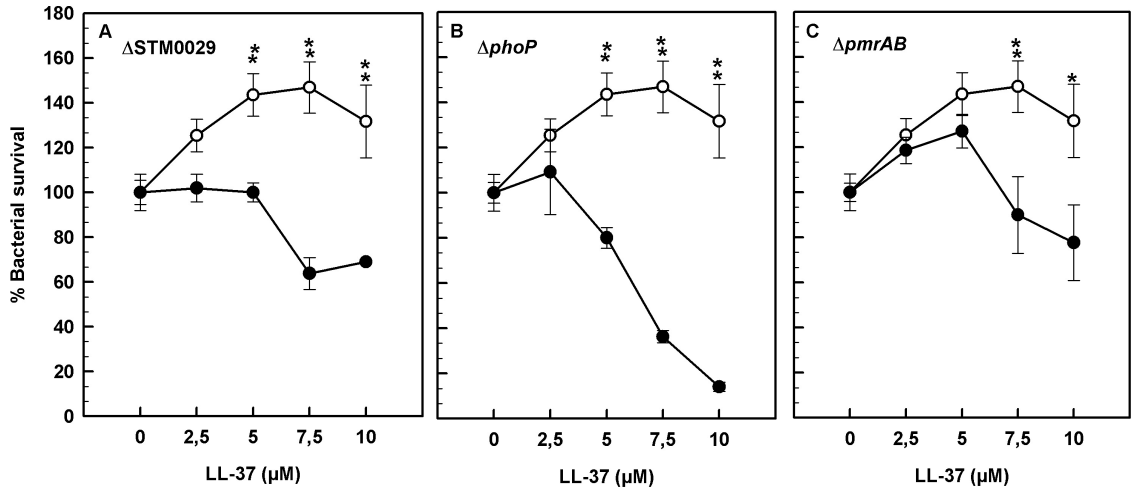


Fig. 6. The ΔSTM0029 strain is sensitive to human cathelicidin LL-37 killing. The wildtype (○, open circles) and mutant strains (●, filled circles) were grown in L-broth to an OD_{600} of approx. 2 and were treated with the indicated concentrations of LL-37. Bacteria were incubated with LL-37 at 37°C for 1 hour and dilutions of the bacterial samples were plated for determination of colony-forming unit (cfu). Percent survival is the mean ± standard deviation (SD) of three independent experiments with similar results. All experiments were performed in triplicate measurements, and error bars indicate standard deviations. *, $P < 0.01$; **, $P < 0.005$.

A number of the mechanisms utilized by *Salmonella* spp against polymyxin B challenges have been well studied. The PmrA/B TCS is currently believed to be one of the major regulatory systems in defense against polymyxin B challenges (Roland et al., 1993). Polymyxin B is also a major component in clinical treatments against multidrug-resistant Gram-positive and Gram-negative bacteria. Therefore, I determined whether STM0029 contributes to resistance against polymyxin B challenges as well. All strains showed susceptibility to polymyxin B, including the wildtype strain (Fig. 7), although clearly the ΔSTM0029, Δ*phoP* and, as expected, the Δ*pmrA*/*B* strain were far more susceptible than the wildtype strain. Taken together, the results indicated that STM0029 is critical for *Salmonella* resistance to both mammalian AMPs and polymyxin B, suggesting the importance of STM0029 for *Salmonella* intracellular survival in host cells.

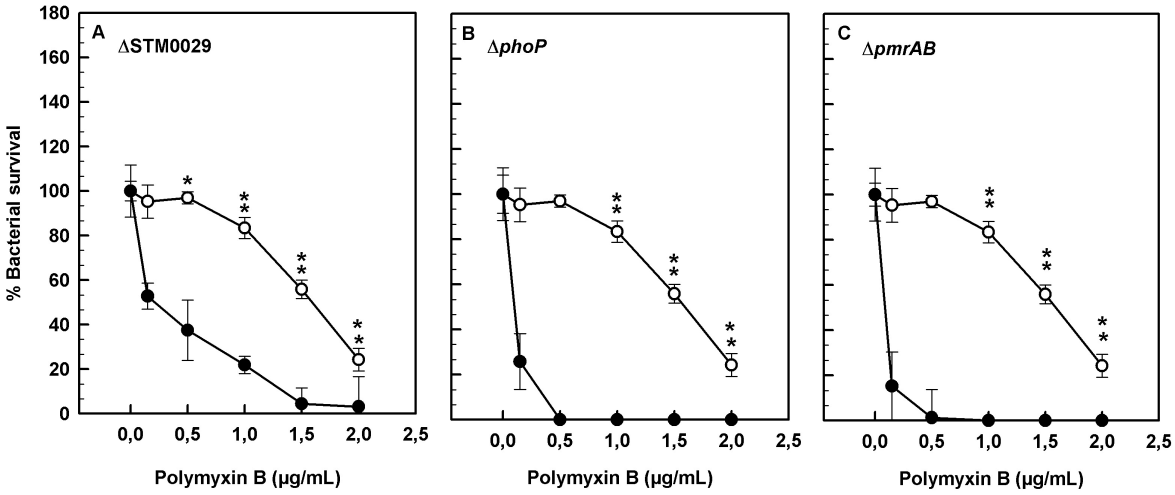


Fig. 7. The ΔSTM0029 strain is sensitive to polymyxin B killing. The wildtype (○, open circles) and mutant strains (●, filled circles) were grown in L-broth to an OD_{600} of approx. 2 and were treated with the indicated concentrations of polymyxin B. Bacteria were incubated with polymyxin B at 37°C for 1 hour and dilutions plated for determination of intracellular colony-forming unit (cfu). Percent survival is the mean ± standard deviation (SD) of three independent experiments with similar results. All experiments were performed triplicate measurements, and error bars indicate standard deviations. *, $P < 0.01$; **, $P < 0.005$.

4.2. Expression of the *Salmonella* STM0029 gene is affected by PmrA/B and not by PhoP/Q

PhoP/Q and the PmrA/B are two component regulatory systems (TCS) involved in *Salmonella* virulence gene expression and intracellular survival (see Introduction). The PhoP/Q TCS in particular has been found to regulate numerous genes involved in the resistance to host bactericidal compound challenges. Since the ΔSTM0029 strain shared some similarity in phenotypes against AMP challenges compared to the Δ*phoP* and the Δ*pmrA/B* strains, I therefore further determined whether expression of STM0029 was associated with the PhoP/Q and/or the PmrA/B TCS regulatory networks. As Mg^{2+} limitations are considered one of the essential signals for the activation of the PhoP/Q and the PmrA/B TCS, strains harbouring transcriptional *lacZ* fusions with STM0029 in the wildtype, the *phoP* and the Δ*pmrA*/*B* mutant backgrounds were grown in either L-broth or defined M9 minimal medium with high (10 mM) or low (50 μM) Mg^{2+} concentrations, and *β*-galactosidase activities were quantified at various time points during the growth phase (Fig. 8, see page 63). The expression of STM0029 in the wildtype strain background showed only low levels of expression, which did not change significantly throughout the growth phase in any of the media tested (Fig. 8A, B, C). The significance of the slight increase observed late in the growth phase in L-broth (Fig. 8A) remains unclear. Essentially the same results were obtained for STM0029 expression in the *phoP* mutant background (Fig. 8E, F, G), indicating that loss of PhoP did not affect expression of STM0029, and independent of the Mg^{2+} concentration in the media. In contrast, a deletion of the *pmrA*/*B* genes resulted in a 50- to 100-fold increased expression of STM0029 in all media used (Fig. 8G, H, I). This surprising observation indicated that rather than a dependence on the PmrA/B TCS for activation and/or expression of STM0029, the PmrA/B regulatory system apparently acts to repress expression of STM0029. These results further indicated that expression of STM0029 was independent of the PhoP/Q TCS, although it remained possible that STM0029 itself might be involved in expression of PhoP/Q-dependent genes in a similar manner as PmrA/B-dependent genes are directly or indirectly regulated by PhoP/Q (Kato and Groisman, 2004).

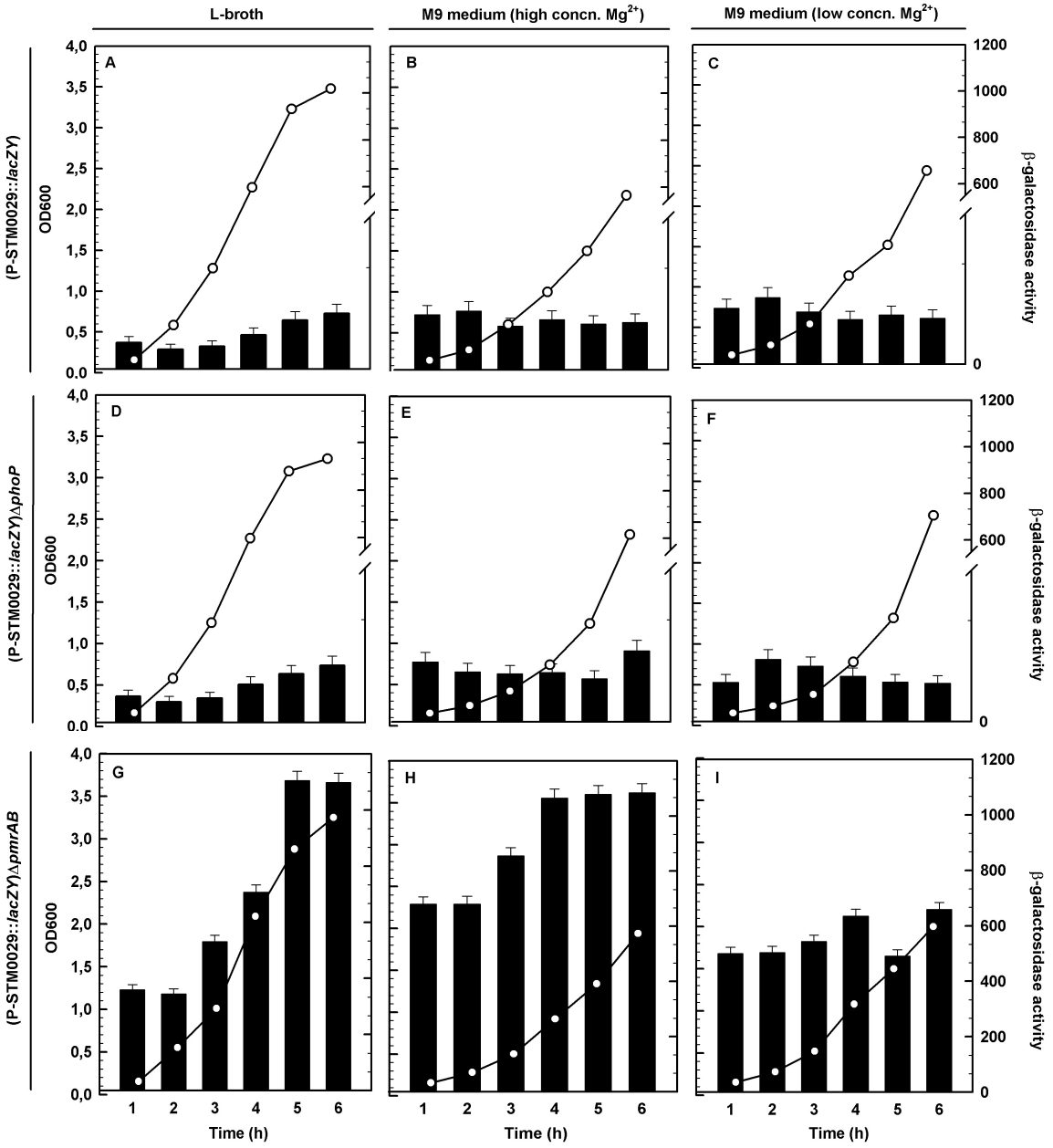


Fig. 8. Expression of STM0029 is repressed by the PmrA/B two component system, but is independent of the PhoP/Q two component system. Strains harbouring transcriptional *lacZ* fusions with the STM0029 gene promoter in either the wildtype (upper row), *phoP* (middle row) or *pmrA*/*B* (bottom row) mutations were grown in L-broth or M9 minimal medium containing high (10 mM) or low (50 μM) concentrations of Mg^{2+}. Bacteria were cultured in baffled glass flasks at 37°C with aeration. The culture growth was recorded by measurement of OD_{600} (circles, left scale). Samples were taken at various time points during culture and processed for quantification of *β*-galactosidase activity (filled bars, right scale). *β*-Galactosidase activity determinations reflecting the promoter activity are reported in Miller units with standard deviations of the means shown (error bars).

In order to determine whether the putative regulator STM0029 might be involved in the regulation of PhoP/Q-dependent genes, I determined the expression of transcriptional fusions of four PhoP-activated genes (*pagP*, *pagD*, *pagC* and *pcgL*) (see introduction 1.3.8.1. for functions of PagP, PagD and PagC, p. 19). PcgL was reported to encode a periplasmic D-alanyl-D-alanine dipeptidase involved in *Salmonella* peptidoglycan synthesis (Hilbert et al., 1999). *β*-Galactosidase activities of transcriptional fusions to indicated genes were measured in the wildtype, the ΔSTM0029, the *phoP* and the Δ*pmrA*/*B* mutant backgrounds, respectively (Fig. 9, see page 65). Expression levels of *pagP* (Fig. 9A), *pagD* (Fig. 9B), *pagC* (Fig. 9C) and *pcgL* (Fig. 9D) in the Δ*phoP* strain were highly reduced compared to the wildtype, consistent with the PhoP-dependence for expression of these genes. In contrast, expression levels of all these genes in the ΔSTM0029 background remained comparable to the wildtype and the Δ*pmrA*/*B* strain background. These results strongly suggested that the reductions in resistance to defensins and polymyxin B were not due to reduced expression of PhoP/Q-dependent genes in the absence of STM0029.

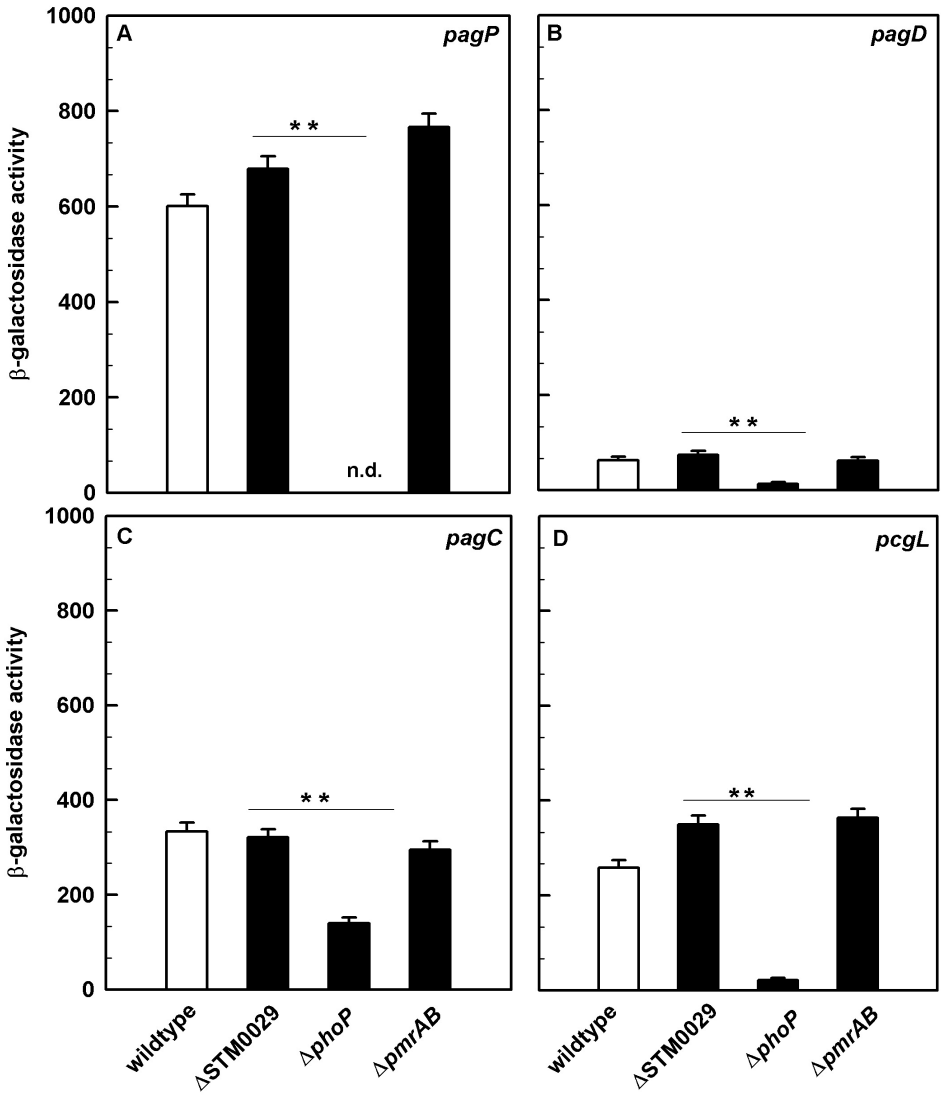


Fig. 9. Expression levels of PhoP/Q-dependent genes are not affected by loss of STM0029. *β*-Galactosidase activities were determined for the indicated transcriptional gene fusions in the wildtype, Δ*phoP*, and Δ*pmrA*/*B* strain backgrounds. The transcriptional activities of the indicated genes in the wildtype strain are shown in open bars, and for the mutant strain backgrounds in filled bars. The data shown are representative of at least three independent experiments. Means for relative units and standard deviations are shown (bars). **, $P < 0.005$.

4.3. Microarray analysis of gene expression affected by STM0029

To gain insight into the molecular basis for differences in pathogenesis between the wildtype and the ΔSTM0029 strain, microarray analyses were performed to compare the global genome expression patterns of the two strains. Strains were grown in either in L-broth or minimal PCN media with low concentrations of inorganic phosphate (Pi) (0.4 mM). Löber et al. (2006) have previously reported that limiting concentrations of Pi is encountered by intracellular *Salmonella* and which is one of the signals which induces expression of the *Salmonella* SPI2 regulon. The gene expression values (normalized fluorescence signal values) were compared to the ΔSTM0029 strain versus the wildtype, where the signal ratio of the ΔSTM0029/wildtype strain yields a relative value of 1.0 when no difference in expression is observed, or 0.0 when the natural log value (base 10) is reported (regarding data acquisition and analysis see Introduction 3.1.3.6. for details, p. 44). Consistent with previous findings (Löber et al., 2006), genes encoded within SPI2 were up-regulated in PCN medium (Data not shown). 891 genes (19.4%) and 569 genes (12.3%) of the *S.* Typhimurium genome revealed significant changes at the transcriptional level in the ΔSTM0029 strain in L-broth (Table IV, see page 80) and 0.4 mM Pi-PCN medium (Table V, see page 81) relative to the wildtype, respectively. Among these genes, great numbers of genes [85 (13.9% of total down-regulated genes) in L-broth and 48 genes (12.5% of total down-regulated) in 0.4 mM Pi-PCN medium] were involved in the function category of "process of cell wall, membrane and envelope biosynthesis" by loss of STM0029.

The category of 20 PhoP/Q-regulated genes was based on the reference of Groisman, E.A. (2001) (Fig. 10A, B, see page 68; Supplementary Table II, see page 137), and were selected for comparison of mRNA expression levels between the ΔSTM0029 strain and the wildtype. In L-broth media, mRNA expression levels of *phoQ* (STM1230), *pmrF* (STM2298), *pgtE* (STM2396), *prgJ* (STM2872) and *mgtC* (STM3764) showed slight increases and the expression level of *ugtL* (STM1601) showed a slight decrease and the *prgI* (STM2873) and *pmrB* (STM4291) genes showed 1.3- and 2-fold decreased expression levels, respectively, in the absence of STM0029 (Fig. 10A, see page 68; Supplementary Tabel II, see page 137). Expression levels of the other PhoP/Q-dependent genes showed no significant differences in expression, consistent with the previous results with the fusion strains (see Fig. 9). Expression levels of *pagP* (STM0628), *pagL* (STM1645) and *pagA* (STM2080), which are involved in the function of LPS lipid A modifcations, were not affected by loss of STM0029. The expression level of *pagD* (STM1244) also showed a slight decrease; however, *pagD* is not considered as one of the PhoP/Q-regulated genes according to a reference from Groisman,

E.A. (2001). In PCN medium, the expression level of *pagA* (STM2080), which is mainly regulated by the PmrA/B TCS showed a slight decrease and expression levels of *prgK* (STM2871), *prgJ* (STM2872) and *prgI* (STM2873) showed increases in the ΔSTM0029 strain (Fig. 10B, see page 68; Supplementary Tabel II, see page 137).

Regarding PmrA/B-regulated genes, 50 genes were chosen based on the *in silico* study reported by Marchal et al. (2004) (Fig. 10A, B, see page 68; Supplementary Table II, see page 139). Among them, ten genes, including *sdhC* (STM0115), *ybjG* (STM0865), *pntA* (STM1479), *yejG* (STM2220), *pgtP* (STM2399), *govA* (STM2982), *yhcN* (STM3361), *glgB* (STM3538), *yibD* (STM3707), *pmrB* (STM4291) and *yjdB* (STM4293) were down-regulated and nine genes, including *leuO* (STM0115), *yceP* (STM1161), *pmrF* (STM2298), *pmrD* (STM2304), STM2400, *stdA* (STM3029), *ipfA* (STM3640) and *slsA* (STM3761) were up-regulated by loss of STM0029 in the L-broth growth condition (Fig. 10A, see page 68; Supplementary Table II, see page 139). In the PCN medium growth condition, *stfA* (STM0195), *yafC* (STM0273) and STM2400 were down-regulated and *ycaR* (STM0987), *pgtP* (STM2399) and *slsA* (STM3761) were up-regulated by loss of STM0029 (Fig. 10B; Supplementary Table II, see page 139).

In summary, the expression of 28 genes involved in the PhoP/Q and the PmrA/B regulatory systems were also affected by loss of STM0029 in the L-broth growth conditions and expression of 11 genes were affected by loss of STM0029 in the PCN medium growth conditions.

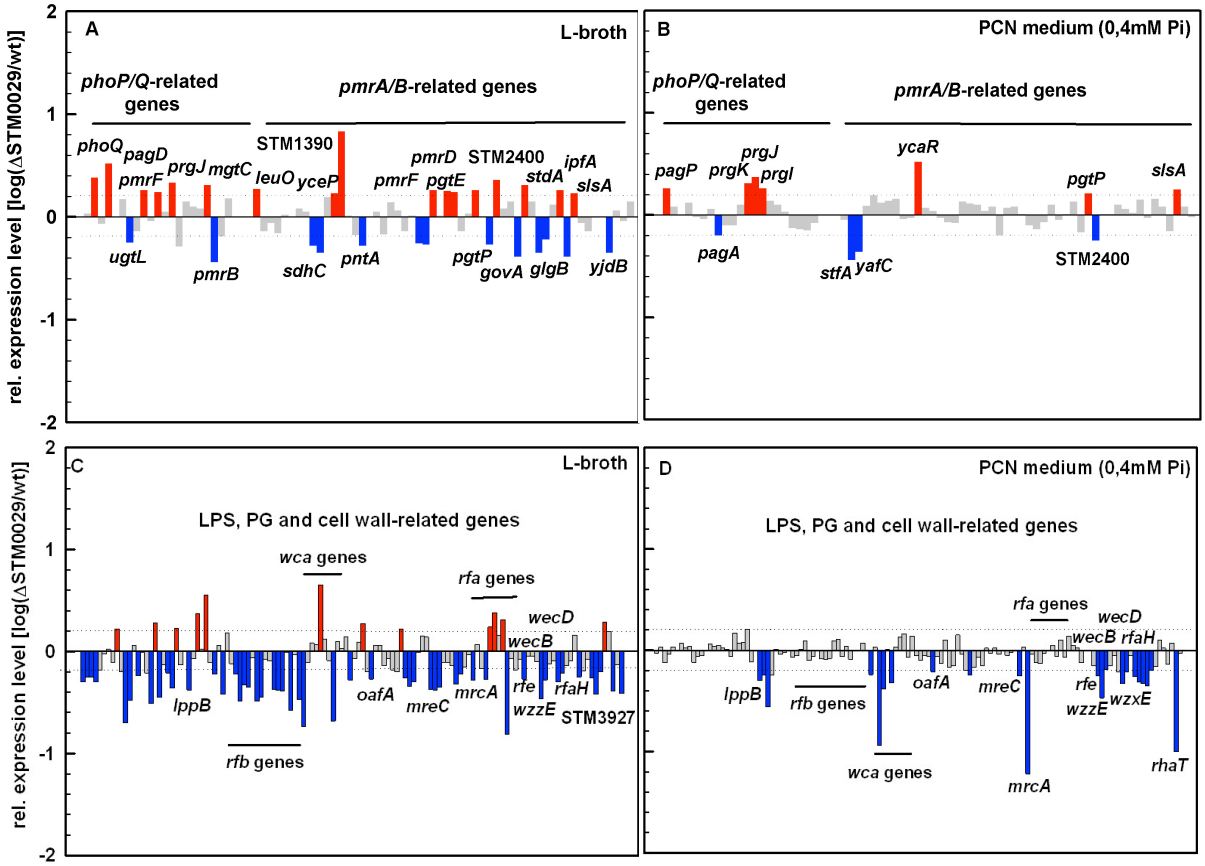


Fig. 10. Microarray analysis of the effect of STM0029 on PhoP/Q-regualted, PmrA/B-regulated and LPS-, PG- and cell wall-associated genes. Bacterial RNA was extracted from bacteria grown in L-broth (A, C) and PCN defined medium with low phosphate (0.4 mM Pi) (B, D), respectively. The relative expression pattern of phoP/Q- and pmrA/B-regualted genes (A, B) as well as LPS, PG and cell wall-associated genes (C, D) are shown. The natural log of hybridisation signal ratios (Δ STM0029 strain/wildtype) are shown in left scales. Expressions of the genes shown in red bars are up-regulated by loss of STM0029; expressions of the genes shown in blue bars are down-regulated by loss of STM0029. The full data on which the figure is based are found in Supplementary Table II.

4.4. STM0029 regulates genes involved in LPS O-antigen biosynthesis and modifications

The results of this study indicated that expression levels of genes involved in the process of cell wall, membrane and envelope biosynthesis showed decreases in expression by loss of STM0029, suggesting that STM0029 is involved in the process of bacterial cell wall biosynthesis and/or modifications. To date, numerous well-studied *Salmonella* virulence genes involved in modifications of LPS lipid A are known to be regulated by the PhoP/Q TCS; however, genes involved in PhoP/Q-mediated LPS lipid A modifications, including *pagP* (STM0628) and *pagL* (STM1465) showed no difference or only slight changes in expression based on the microarray results (Fig. 10A, B) and MALDI-TOF studies of LPS (data not shown). Interestingly, the expression of numerous genes involved in LPS O-antigen biosynthesis and modifications showed decreases after loss of STM0029, including *rfbM* (STM2084, synthesis of GDP-rhamnose), *rfbX* (STM2088, assembly or transfer of LPS O units), *rfbJ* (STM2089, synthesis of CDP-abequose), *oafA* (STM2232, acetylation of the O-antigen), *mreC* (STM3921, rod shape-determining protein), *wzzE* (STM3919, lipopolysaccharide biosynthesis protein), *wecB* (STM3920, reversible interconversion of UDP-GlcNAc and UDP-ManNAc), *wecD* (STM3924, glucose-1-phosphate thymidylyltransferase), *wzxE* (STM3926, TDP-4-oxo-6-deoxy-D-glucose transaminase), STM3927 (O-antigen translocase in LPS biosynthesis) and *rfaH* (STM3977, biosynthesis of lipopolysaccharide core) in L-broth (Fig. 10C, D, see page 68; Table VI, see page 82). In the PCN medium growth conditions, the expression of genes, including *wcaF* (STM2110, putative acyltransferase), *wcaE* (STM2111, glycosyltransferase 2 family enzymes), *oafA*, *mreC*, *rfe* (STM3918, undecaprenyl-phosphate N-acetylglucosaminyltransferase), *wzzE*, *wecB*, *wecD*, *wzxE* and *rhaT* (STM4050, L-rhamnose-H^+ transport protein) showed decreases after loss of STM0029 (Fig. 10C, D, see page 68; Table VI, see page 82).

In addition to bacterial LPS, the peptidoglycan layer, which forms a barrier between the cytoplasm and the outer membrane, is also a target for certain antibiotics and host pattern recognition molecules as well (reviewed by Davis and Weiser, 2011). According to results of this study, the expression of a number of genes involved in the peptidoglycan biosynthesis were affected by loss of STM0029 as well, including *lppB* (STM1376, putative methyl-accepting chemotaxis protein) and *mrcA* (STM3493, peptidoglycan synthetase) (Table VI, see page 82).

In order to confirm results obtained from the microarray analysis, I chose three genes (*oafA*, *rfaH* and *rfe*), which are critical for LPS O-antigen biosynthesis and modifications to verify their expression levels by quantitative RT-PCR using total RNA and cDNA isolated

from cultures grown in L-broth (Fig. 11A) and 0.4 mM-Pi PCN medium (Fig. 11B). The *dnaA* gene was chosen as an internal control, since its expression obtained from microarray analyses showed no difference in both L-broth (rel. mRNA expression level [log -0.04]) and in PCN medium (rel. mRNA expression level [log -0.05]) between the wildtype and the ΔSTM0029 strain. Relative mRNA expression levels of *oafA*, *rfaH* and *rfe* exhibited decreases in expression in the DSTM0029 strain compared to the wildtype, in particular, *oafA* showed more than an 80% reduction in expression under both growth conditions.

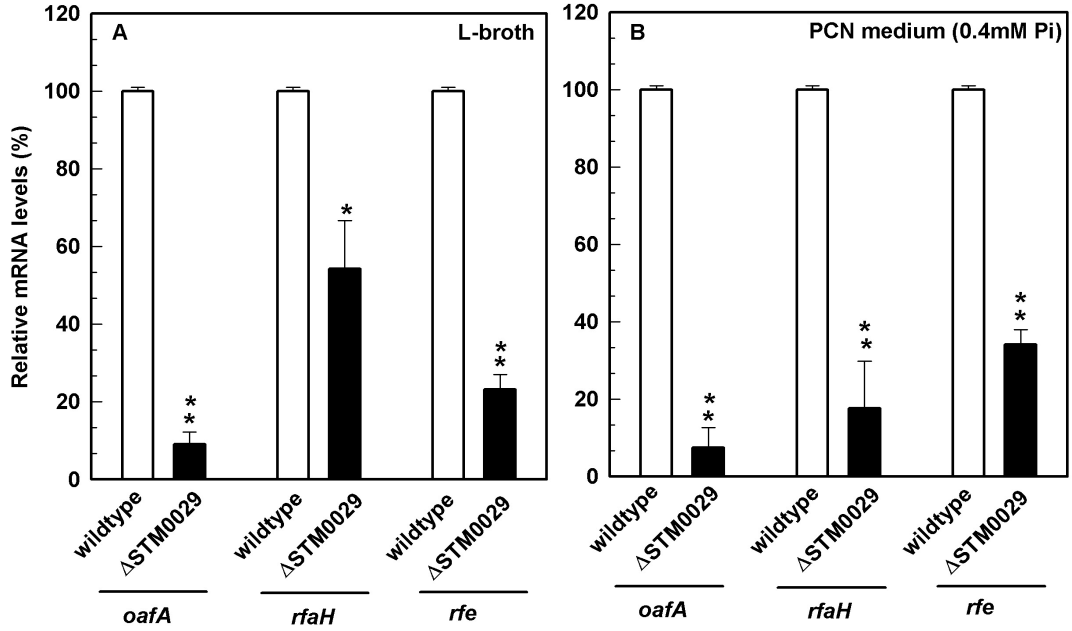
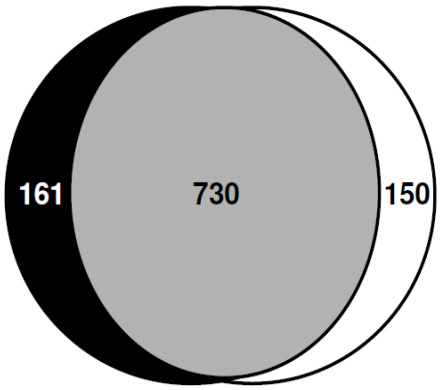


Fig. 11. Loss of STM0029 affects expressions of genes involved in LPS biosynthesis and modifications. Bacterial RNA was extracted from cells grown in L-broth (A) or PCN defined medium with low concentrations phosphate (0.4 mM Pi) (B). The bar chart shows the relative levels of *oafA*, *rfaH* and *rfe* mRNA in the STM0029 deletion mutant (filled bars) compared to the wildtype strain which was set at 100% (open bars) as determined by quantitative real-time PCR using the *dnaA* gene transcript as an internal control. **, $P < 0.005$.

4.5. Transcriptomic profile reveals genes specifically regulated by STM0029

Based on the *in vitro* β-galactosidase analyses (Fig. 8G, H, I), the expression of STM0029 was found to be strongly repressed by the PmrA/B TCS. Furthermore, the expression levels of additional PmrA/B-related genes, including *pmrF* (STM2298), *pmrD* (STM2304) and *pmrB* (STM4291) were also affected by loss of STM0029 in L-broth (Supplementary Table II, see page 139), suggesting that STM0029 might be associated with the PmrA/B regulatory system. In order to determine whether STM0029 is directly involved in the PmrA/B regulatory cascade or serves as a co-factor, which functions in regulatory networks in addition to the PmrA/B regulatory system, I compared the transcriptomic profiles obtained in four different experimental strains, including the wildtype, the ΔSTM0029, the Δ*pmrA*/*B* and the ΔSTM0029/Δ*pmrA*/*B*-double mutant strains in order to determine the full set of genes regulated specifically by STM0029. Genes which showed the same or similiar (≥ 2-fold changes) relative mRNA expression levels in both the ΔSTM0029 and the ΔSTM0029/Δ*pmrA*/*B*-double deletion strain were considered as specifically STM0029-regulated genes. As shown in the figure 12A (see page 72), the expression of 730 genes were found to be co-regulated by both STM0029 and PmrA/B. In contrast, the expression of 150 genes were only affected by loss of the PmrA/B; and the expression of a total of 161 genes (3.5% of the *Salmonella* genome) were specifically affected by loss of STM0029 (Table VII, see page 83). Among these 161 genes, 112 genes showed reduced expression in the absence of STM0029. Based on categories in the Clusters of Orthologous Groups of proteins (COG) database, proteins encoded by these 112 genes were divided into 19 groups (Fig. 12B, see page 72). The first large group (category) was assigned as the category "Function unknown" (14.3%). The second and the third group was aassigned as the category "General function prediction only" (13.4%) and "Cell wall/membrane/envelop biogenesis" (13.4%), respectively. Interestingly, the *oafA* gene, which is involved in LPS O-antigen acetylation was found in the category "Cell wall/membrane/envelop biogenesis" as a specific, STM0029-regulated gene (Table VIII, see page 84).

A



B

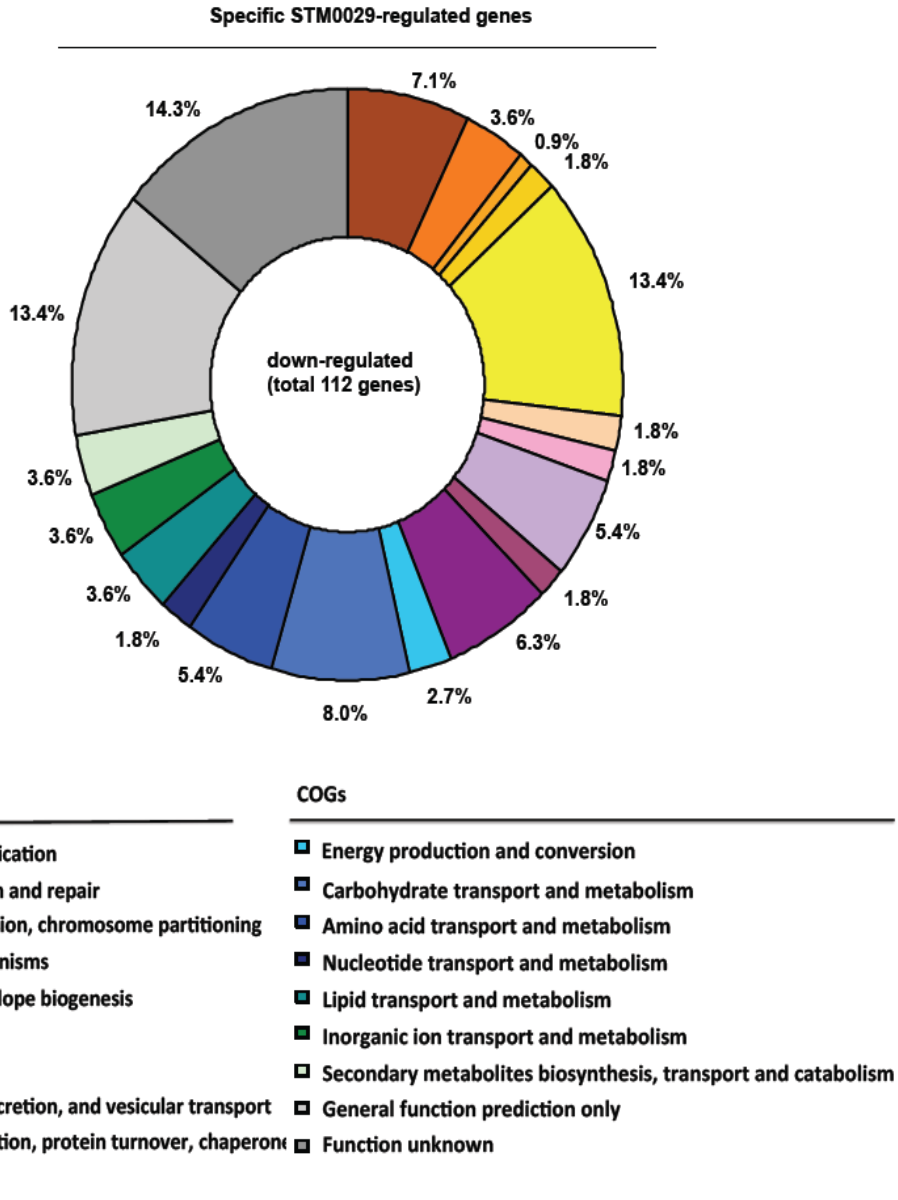


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Fig. 12. Transcriptomic profile of the genes specifically regulated by STM0029 in *S.* Typhimurium. Bacterial RNA was extracted from the wildtype, the ΔSTM0029, the Δ*pmrA/B* and the ΔSTM0029/Δ*pmrA/B* double mutant strains grown in L-broth. Candidate genes whose expressions were only affected in the ΔSTM0029 mutant background were selected by comparison to genes, whose expressions were affected under the Δ*pmrA/B*- and ΔSTM0029/Δ*pmrA/B*-deleted backgrounds. (A) The pie chart indicates numbers of the genes selected from the microarray analysis. The portion with the black background represents the number of the genes specifically regulated by STM0029 (left). The portion with the gray background represents the number of the genes co-regulated by STM0029 and the PmrA/B two component system (middle). The portion with the white background represents the number of the genes specifically regulated by the PmrA/B two component system (right). (B) The pie chart indicates the relative abundance of categories of the genes with reduced expressions affected specifically by loss of STM0029. Colours correspond to categories in the COG database. A detailed list of the genes are found in Table VI.

4.6. Role of *Salmonella* STM0029 in resistance to human and mouse sera

Based on results obtained from microarray analyses and quantitative RT-PCR, the expression of *oafA* showed a reduction in expression after loss of STM0029. OafA belongs to a member of a family of integral membrane trans-acylases and it is involved in acetylation of *Salmonella* LPS O-antigen (Slauch et al., 1995). Acetylation is a post-translational modification believed to be important for host-microbes interactions (Ribet and Cossart, 2010). For instance, *Listeria monocytogenes* is able to induce deacetylation of histone H4 during early phases of infection in order to assist its pathogenesis (Hamon et al., 2007). Moreover, a deletion of *oafA* in *Heamophilus influenzea* has also been reported to result in a loss of the resistance againt host serum challenges (Fox et al., 2005).

Serum opsonisation *via* the complement binding is required for activation of the host innate immune response to eliminate invasive pathogens (Heffernan et al., 1992; Joiner at al., 1982; Stemmer et al., 1985), and pathogens have evolved various mechanisms against complement challenges. The proper distribution of O-antigen chain length/modifications has been reported to be required for complement resistance in *Salmonella* spp (Morona et al., 1995; Murray et al., 2003). McConnel and Wright (1979) reported that the growth temperature affected the size and the distribution of *Salmonella anatum* LPS O-antigen. Murray et al. (2006) have indicated that incubation of *S.* Typhimurium in heat-inactivated guinea pig serum induced the production of very-long O-antigen (VL-OAg), which conferred a higher level of survival against serum killing. However, little is known about the regulation of LPS O-antigen and its effect on serum resistance. I therefore tested the sensitivity of the ΔSTM0029 strain against human and mouse sera compared to the wildtype (Fig. 13, see page 75). The wildtype and the ΔSTM0029 strains were grown to early stationary phase (OD_{600} approx. 2.0) and incubated with 100% normal non-heated human/mouse sera (NNS) or 100% heat-inactivated human/mouse sera (HIS) at 37°C for one hour, and the relative ratio of survival was determined as described in Materials and Methods (see section 3.3.3., p. 49). The ΔSTM0029 strain showed a 60% and a 50% reduction in survival compared to the wildtype strain after human and mouse serum challenges, respectively.

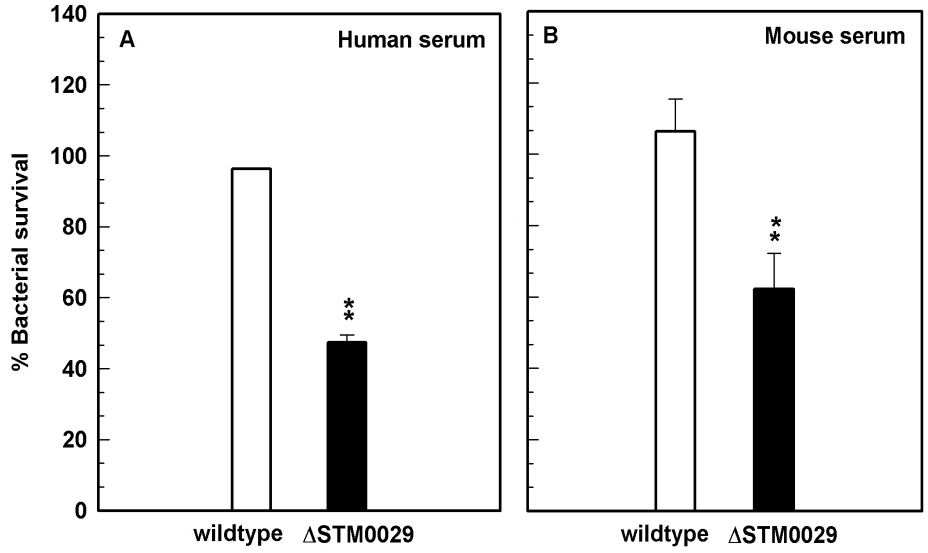

Fig. 13. The ΔSTM0029 strain is susceptible to human and mouse sera. The wildtype (open bars) and the ΔSTM0029 strain (filled bars) were incubated with 100% normal non-heated human/mouse sera (NNS) or heat-inactivated human/mouse sera (NIS), respectively. Bacteria were grown till OD_{600} approx. 2 and incubated with NNS and NIS at 37°C for 1 hour, respectively. Dilutions were plated for determination of intracellular colony-forming unit (cfu). The ratio of survival of NNS and NIS was calculated with the formula 100 × [mean (cfu ml^{-1} in NNS/cfu ml^{-1} in HIS) of mutant]/[mean (cfu ml^{-1} in NNS/cfu ml^{-1} in HIS) of wildtype]. Percent survival is the mean ± standard deviations (SD) of three independent experiments with similar results. All experiments were performed triplicate measurements, and error bars indicate standard deviations. **, $P < 0.005$.

4.7. Alignments of the *Salmonella* putative transcriptional regulator STM0029 amino acid sequence and promoter region analysis

According to results obtained from AMP killing assays and microarray analyses, I found that the putative transcriptional regulator STM0029 is important for *Salmonella* resistance against host bactericidal challenges through the regulation of the genes involved in LPS O-antigen and peptidyglycan biosynthesis and modifications. In order to determine possible homologues existing in other pathogenic and/or non-pathogenic bacterial strains, I therefore performed alignments based on the *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 STM0029 amino acid sequence provided by the NCBI database (http://www.ncbi.nlm.nih.gov/protein/16763419?). 100 different bacterial species which share similarities of the amino acid sequence to the *Salmonella* putative transcriptional regulator STM0029 were found based on the NCBI BLAST algorithm (http://blast.ncbi.nlm.nih.goc/Blast.cgi). Among which, a total of 15 strains with identical amino acid sequences all belonged to *Salmonella* spp. (Table IX, see page 86). However, in the same Family (*Enterobacteriaceae*) as *Salmonella* spp, no other Genera of bacteria were found to have near-identical amino acid sequence to the *Salmonella* STM0029 protein. This observation suggested that the putative transcriptional regulator STM0029 should be *Salmonella*-specific. For the low-homology homologues, the STM0029 amino acid sequence showed similarity to the transcriptional regulator ToxR in *Erwinia pyrifoliae* Ep1/96 (identity, 35%; similarity, 46%), *Erwinia tasmaniensis* Et1/99 (identity, 38%; similarity, 55%), *Vibrio cholerae* HC-02A1 (identity, 34%; similarity, 5%8), *Vibrio cholerae* 12129(1) (identity, 34%; similarity, 58%) and the CadC family transcriptional regulator in *Rahnella* spp Y9602 (identity, 47%; similarity, 69%), *Pantoea* spp aB (identity, 32%; similarity, 57%), *Burkholderia* spp Ch1-1 (identity, 34%; similarity, 56%), *Enterobacter asburiae* LF7a (identity, 33%; similarity, 54%) and *Burkholderia xenovorans* LB400 (identity, 33%; similarity, 55%). In *V. cholera*, ToxR is a transmembrane regulatory protein required for the synthesis of cholera toxin (Miller et al., 1984). Mathur and Waldor (2004) reported that ToxR controls the resistance to bactericidal/permeability-increasing (BPI), an antimicrobial protein, by regulating the production of the outer membrane protein OmpU. In *Escherichia coli*, CadC, which belongs to the ToxR-like protein family (Miller et al., 1987), is a membrane-integrated positive transcriptional activator (Watson et al., 1992). CadC is located at an upstream of the *cadBA* operon and encodes a 58 kDa inner membrane protein (Küper and Jung, 2005; Watson et al., 1992). Currently, the Cad proteins are believed to be involved in the expression of genes associated with colonization, and production of polyamines

associated with protection of some pathogenic *Escherichia coli* against stress conditions (Torres, 2009). Similiarity to ToxR and CadC might emphasize the importance of the *Salmonella*-specific gene, STM0029, to *Salmonella* pathogenesis as well.

I further compared the conserved protein domain of the *Salmonella* STM0029 gene to the full list of conserved domains, which are present in the current proteins as determined by a Conserved Domain Database search using RPS-BLAST. This dataset includes all domain models that meet or exceed the RPS-BLAST threshold for statistical significance, as well as the conserved domain superfamilies to which they belong. The *Salmonella* STM0029 protein harbours a conserved domain, which belongs to the Helix-turn-helix_XRE Superfamily (HTH_XRE) (Accession: cl00088). HTH_XRE-family-like proteins are prokaryotic DNA binding proteins belonging to the xenobiotic response element family of transcriptional regulators. Proteins which are known to be involved in this family have functions in bacterial plasmid copy control, bacterial methylases, various bacteriophage transcription control proteins and a vegetative specific protein from *Dictyostelium discoideum* (Slime mould) based on the SMART (Simple Modular Architecture Research Tool) mode description (http://smart.emblheidelberg.de/smart/do_annotation.pl?ACC=SM00530&BLAST=DUMM).

Moreover, the promoter region of STM0029 was analyzed as well to predict possible transcriptional factor binding sites (Fig. 14, see page 79). The positive sense of the sequence (369 bases; the sequence with the yellow background) between the STM0029 start codon (the CAT codon with the red underline) and STM0030 start codon (the ATG codon with the black underline) was chosen for further analyses. The open source BPROM (Prediction of bacterial promoters) (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) was chosen to analyze the possible -10 and -35 boxes. BPROM (Softberry) is a bacterial sigma70 promoter recognition program with about 80% accuracy and specificity. It is best used in regions immediately upstream from the open reading frame start for an improved gene and operon prediction in bacteria (a list of references with BPROM applications, see the link: http://linux1.softberry.com/publ_topic.htm#BPROM). As shown in Fig. 14, putative -10 and -35 boxes were predicted as TTATAAAAT (the purple codon) and GTGCCA (the blue codon), respectively. Furthermore, the indicated promoter region of STM0029 was also analyzed by the Virtual Footprint programme Version 3.0 (http://www.prodoric.de/vfp/) (Münch, et al., 2005) to find out transcriptional factors, which might possibly bind on the STM0029 promoter region. 31 transcriptional factors from the database were shown to possibly bind on the STM0029 promoter region. Among them, OmpR (Kanamaru et al., 1989) and OxyR (Christman et al., 1989) were two transcriptional

factors displayed the highest score compared to other transcriptional factors. OmpR is a response regulator involved in the OmpR/EnvZ TCS that responds to osmotic stresses (see introduction, p. 22). OxyR acts as a positive regulator for hydrogen peroxide-inducible genes in *E. coli* and *S.* Typhimurium. Taken together, this finding suggested that STM0029 might have high potential to play serve as a transcriptional regulator, *e.g.* ToxR and CadC and/or be regualed by other well-unknown transcriptional regulators, *e.g.* OmpR and OxyR.

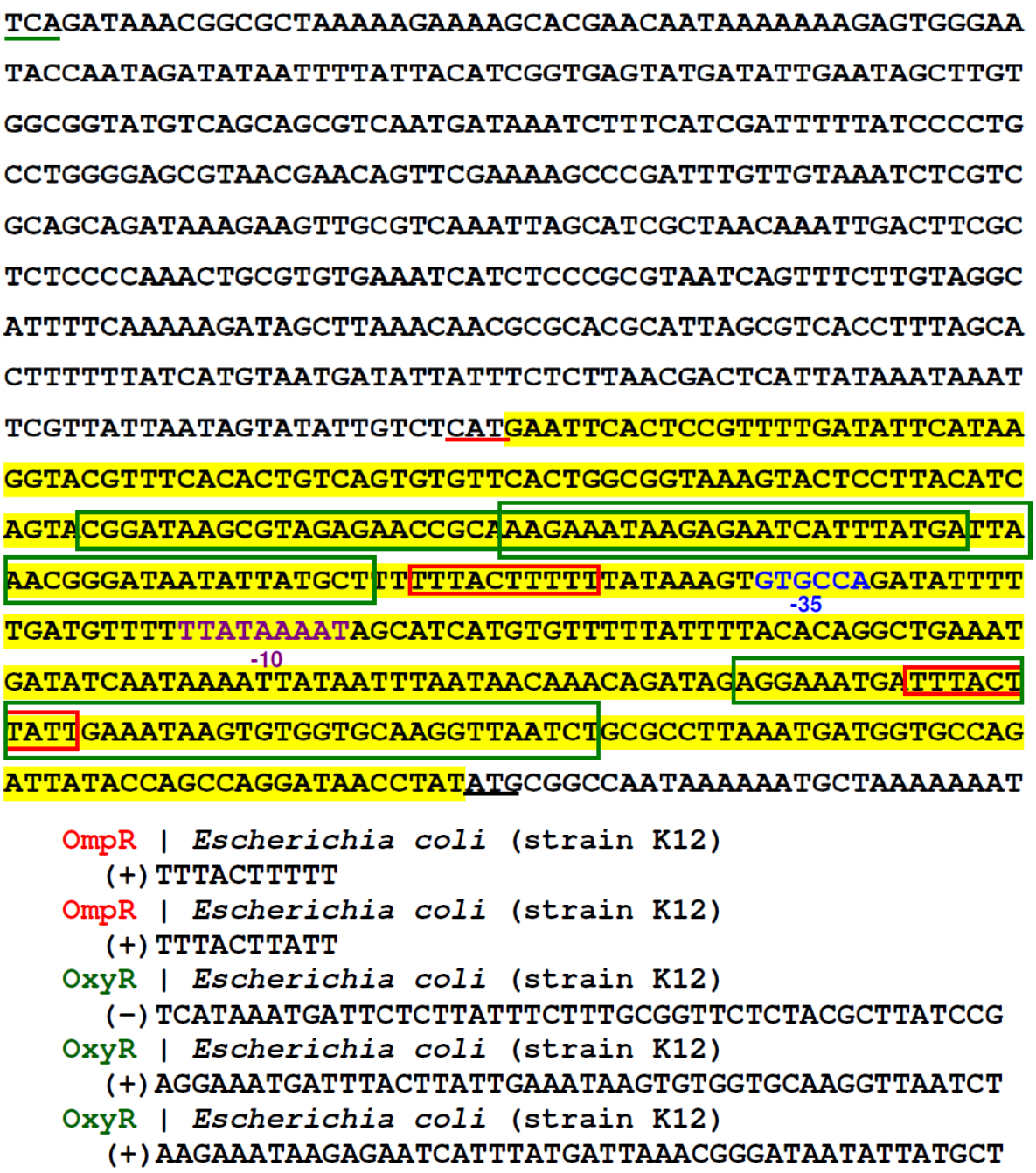

Fig. 14. Analysis of the predicted STM0029 promoter region. The Virtual Footprint programme Version 3.0 was used to analyze the STM0029 promoter region based on the positive sense of the gene sequence. The sequence in the yellow background indicates the predicted STM0029 promoter region. Underlined (CAT, red) bases represents the start codon of the STM0029 gene; underlined (TCA, green) bases represents the stop codon of the STM0029 gene; underlined (ATG, black) bases represents the start codon of the STM0030 gene. Predicted -10 and -35 boxes were indicated in the purple colour and the blue colour, respectively. Red and green enclosed boxes represent binding domains of the transcription factors OmpR and OxyR, respectively. (+) indicates the positive sense of the transcriptional factor binding sequence; (-) indicates the negative sense of the transcriptional factor binding sequence.

Table IV. Genes affected by loss of STM0029 in L-broth (rel. expression level [Δ STM0029/wildtype])[a]

COG Description	No. of genes	
	up-regulated	down-regulated
Information storage and processing		
Translation, ribosomal structure and biogenesis	3	4
RNA processing and modification	3	24
Transcription	1	1
Replication, recombination and repair	3	13
Chromatin structure and dynamics		
Cellular processes and signaling		
Cell cycle control, cell division, chromosome partitioning	1	8
Nuclear structure		
Defense mechanisms	5	5
Signal transduction mechanisms	5	20
Cell wall/membrane/envelope biogenesis	4	85
Cell motility	7	26
Cytoskeleton		2
Extracellular structures	8	18
Intracellular trafficking, secretion, and vesicular transport	6	9
Posttranslational modification, protein turnover, chaperones	9	16
Regulation	28	76
Metabolism		
Energy production and conversion	4	14
Carbohydrate transport and metabolism	33	70
Amino acid transport and metabolism	27	34
Nucleotide transport and metabolism	4	11
Coenzyme transport and metabolism	3	7
Lipid transport and metabolism	11	14
Inorganic ion transport and metabolism	30	41
Secondary metabolites biosynthesis, transport and catabolism	13	34
Poorly characterised		
General function prediction only	49	54
Function unknown	23	25
Amount	280	611

[a] Genes, whose expressions showed the log of hybridization signal ratios (rel. expression level [Δ STM0029/wildtype]) ≥ 0.33 were selected.

Table V. Genes affected by loss of STM0029 in PCN medium (rel. expression level [Δ STM0029/wildtype])[a]

COG Description	No. of genes	
	up-regulated	down-regulated
Information storage and processing		
Translation, ribosomal structure and biogenesis		1
RNA processing and modification	7	11
Transcription		
Replication, recombination and repair	3	4
Chromatin structure and dynamics		1
Cellular processes and signaling		
Cell cycle control, cell division, chromosome partitioning	1	3
Nuclear structure		
Defense mechanisms	5	4
Signal transduction mechanisms	1	9
Cell wall/membrane/envelope biogenesis	13	48
Cell motility	2	11
Cytoskeleton		1
Extracellular structures		5
Intracellular trafficking, secretion, and vesicular transport		6
Posttranslational modification, protein turnover, chaperones	4	13
Regulation	16	20
Metabolism		
Energy production and conversion	3	15
Carbohydrate transport and metabolism	30	60
Amino acid transport and metabolism	8	17
Nucleotide transport and metabolism	1	6
Coenzyme transport and metabolism	3	7
Lipid transport and metabolism	5	9
Inorganic ion transport and metabolism	5	9
Secondary metabolites biosynthesis, transport and catabolism	10	26
Poorly characterised		
General function prediction only	25	47
Function unknown	42	52
Amount	184	385

[a] Genes, whose expressions showed the log of hybridization signal ratios (rel. expression level [Δ STM0029/wildtype]) ≥ 0.33 were selected.

Table VI. List of LPS O-antigen and PG-related genes regulated by STM0029 compared to the wildtype[a, b]

No.	Gene	Predicted functions (or Homolog)	Fold Change		Reference
			L-broth	PCN [c]	
STM1376	*lppB*	Putative methyl-accepting chemotaxis protein	-2.5	-2.0	Fadl et al., 2005
STM2084	*rfbM*	Synthesis of GDP-rhamnose from mannose-1-phosphate	-3.3	-0.7	Jiang et al., 1991
STM2088	*rfbX*	Assembly or transfer of LPS O unit	-3.3	-0.6	Reeves, 1993
STM2089	*rfbJ*	Synthesis of CDP-abequose	-3.0	-0.5	Reeves, 1993
STM2110	*wcaF*	Biosynthesis of the extracellular polysaccharide colanic acid	0.5	-6.2	Ophir et al., 1994
STM2111	*wcaE*	Glycosyltransferase 2 family enzymes involved in O-antigen biosynthesis	0.5	-2.5	
STM2232	*oafA*	Acetylation of the O-antigen (LPS)	-1.3	-1.4	Slauch et al., 1995 Slauch et al., 1996
STM3373	*mreC*	Rod shape-determining protein	-2.1	-1.7	Costa et al., 1993
STM3493	*mrcA*	Peptidoglycan synthetase	-1.0	-8.1	
STM3918	*rfe*	Undecaprenyl-phosphate N-acetylglucosaminyltransferase	-0.7	-1.7	Keenleyside et al., 1994
STM3919	*wzzE*	Enterobacterial common antigen (ECA) polysaccharide chain length modulation protein	-3.1	-3.1	
STM3920	*wecB*	Reversible interconversion of UDP-GlcNAc and UDP-N-acetylmannosamine (UDP-ManNAc)	-1.9	-1.3	Barua et al., 2002
STM3924	*wecD*	Glucose-1-phosphate thymidylyltransferase	-1.7	-1.3	Hung et al., 2006 Ramos-Morales et al., 2003
STM3926	*wzxE*	TDP-4-oxo-6-deoxy-D-glucose transaminase	-2.5	-2.0	
STM3927		O-antigen translocase in LPS biosyntesis	-3.3	-0.7	
STM3977	*rfaH*	Transcriptional activator affecting biosynthesis of lipopolysaccharide core	-3.0	-0.5	Kong et al., 2009
STM4050	*rhaT*	L-rhamnose-proton symport	0.5	-6.2	Al-Zarban et al., 1984 Tate et al., 1992

[a] Genes whose expression showed at least a two-fold difference (the median natural logarithm ≥ 0.33) were shown.

[b] Protein encode by differentially expresssed genes were identified by annotation or homology to known proteins (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome) and assigned to categories based on description in clusters of orthologous groups of proteins (COGs).

[c] PCN medium contains 0.4 mM Pi.

Table VII. Genes specifically regulated by STM0029 in L-broth[a] (rel. expression level [Δ STM0029/wildtype])[b]

COG Description	No. of genes	
	up-regulated	down-regulated
Information storage and processing		
Translation, ribosomal structure and biogenesis		
RNA processing and modification		8
Transcription		
Replication, recombination and repair		4
Chromatin structure and dynamics		
Cellular processes and signaling		
Cell cycle control, cell division, chromosome partitioning		1
Nuclear structure		
Defense mechanisms		
Signal transduction mechanisms		2
Cell wall/membrane/envelope biogenesis	3	15
Cell motility		2
Cytoskeleton		
Extracellular structures		2
Intracellular trafficking, secretion, and vesicular transport		6
Posttranslational modification, protein turnover, chaperones	2	2
Regulation	3	7
Metabolism		
Energy production and conversion	2	3
Carbohydrate transport and metabolism	13	9
Amino acid transport and metabolism	4	6
Nucleotide transport and metabolism	1	2
Coenzyme transport and metabolism	2	
Lipid transport and metabolism	2	4
Inorganic ion transport and metabolism	3	4
Secondary metabolites biosynthesis, transport and catabolism		4
Poorly characterised		
General function prediction only	3	15
Function unknown	11	16
Amount	49	112

[a] Genes, whose expressions showed same or similiar (≥ two-fold changes) relative mRNA expression levels in both Δ STM0029 and Δ STM0029/Δ*pmrA/B*-double deletion conditions were chosen as considering as specifically STM0029-regulated genes.

[b] Genes showing the log of hybridization signal ratios (rel. expression level [Δ STM0029/wildtype]) ≥ 0.33 were selected.

Table VIII. Specific STM0029-regulated genes involved in cell membrane proteins[a,b,c]

No.	Gene	Predicted functions (or Homolog)	Fold Change	Reference
L-broth				
LPS (2)				
STM0776	*galE*	UDP-glucose 4-epimerase	-1.7	
STM2232	*oafA*	Acetylation of the O-antigen (LPS)	-2.0	Slauch et al., 1995 Slauch et al., 1996
Outer membran protein (9)				
STM0282		Paralog of *E. coli* putative outer membrane protein	-1.9	
STM0687	*ybfM*	Putative outer membrane protein	-1.5	
STM0816	*ybhS*	Putative ABC superfamily (membrane) transport protein	-1.4	
STM0833	*ompX*	Outer membrane protease, receptor for phage OX2	-1.7	Hu et al., 2009
STM1254		Putative outer membrane lipoprotein	-3.1	
STM1504	*ynaI*	Putative integral membrane protein	-2.2	
STM2685	*smpA*	Small membrane protein A	-2.1	Lewis et al., 2008
STM4051		Putative outer membrane protein	-2.1	
STM4223	*yjbF*	Putative outer membrane lipoprotein	-4.1	Christopherson et al., 2008
Periplasmic protein (6)				
STM0708	*ybfA*	Putative periplasmic protein	-2.0	
STM1123		Putative periplasmic protein	-4.2	
STM1503	*ynfB*	Putative periplasmic protein	-1.6	
STM1897	*yebB*	Putative periplasmic protein	-1.5	
STM2148		Putative periplasmic protein	-1.7	
STM2156		Hypothetical 16.9 kd lipoprotein precursor	-1.8	
Inner membrane protein (6)				
STM1504	*ynfA*	Putative inner membrane lipoprotein	-1.5	
STM2779		Putative inner membrane protein	-2.0	
STM3761	*slsA*	Pathogenicity island encoded protein: SPI3; SlsA; putative inner membrane protein	-1.8	
STM3774		Putative inner membrane protein	-1.5	
STM4332	*yjeJ*	Putative inner membrane protein	-3.1	
STM4599	*yjjY*	Putative inner membrane protein	-1.5	

[a] Genes whose expression showed at least a two-fold difference (the median natural logarithm ≥ 0.33) were shown.

[b] Genes, which showed same or similiar (≥ 2-fold changes) relative mRNA expression levels in both ΔSTM0029 and ΔSTM0029/Δ*pmrA/B*-double deletion conditions were chosen as considering as specifically STM0029-regulated genes.

[c] Protein encode by differentially expresssed genes were identified by annotation or homology to known proteins (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome) and assigned to categories based on description in clusters of orthologous groups of proteins (COGs).

Table IX. List of *Salmonella* spp with identical STM0029 proteins (amino acid sequences)[a]

Strains	Locus_tag	Accession	Predicted functions	Reference
Salmonella enterica subsp. *enterica* serovar Typhimurium str. UK-1	STMUK_0030	AEF05964.1	Putative transcriptional regulator	Luo et al., 2011
Salmonella enterica subsp. *enterica* serovar Typhimurium str. ST4/74	STM474_0031	ADX15777.1	Putative transcriptional regulator	Richardson et al., 2011
Salmonella enterica subsp. *enterica* serovar Typhimurium str. TN061786	SEE_04593	EFX47474.1	Putative transcriptional regulator	
Salmonella enterica subsp. *enterica* serovar Typhimurium str. T000240	STMDT12_C00300	BAJ34973.1	Putative transcriptional regulator	Izumiya et al., 2010
Salmonella enterica subsp. *enterica* serovar Typhimurium str. SL1344	SL1344_0030	CBW16131.1	Putative transcriptional regulator	
Salmonella enterica subsp. *enterica* serovar Typhimurium str. 14028S	STM14_0037	ACY86574.1	Putative transcriptional regulator	Jarvik et al., 2010
Salmonella enterica subsp. *enterica* serovar Typhimurium str. D23580	STMMW_00291	CBG23048.1	Putative transcriptional regulator	Kingsley et al., 2009
Salmonella enterica subsp. *enterica* serovar Hadar str. RI_05P066	SeH_A0245	EDZ35345.1	Conserved hypothetical protein	
Salmonella enterica subsp. *enterica* serovar 4,[5],12:i:- str. CVM23701	SeI_A4349	EDZ18076.1	Conserved hypothetical protein	
Salmonella enterica subsp. *enterica* serovar Saintpaul str. SARA23	ZP_03163308.1	ZP_03163308.1	Conserved hypothetical protein	
Salmonella enterica subsp. *enterica* serovar Saintpaul str. SARA23	SeSPA_A0534	EDY24109.1	Conserved hypothetical protein	
Salmonella enterica subsp. *enterica* serovar Hadar str. RI_05P066	SeH_A0245	ZP_02684280.1	Conserved hypothetical protein	
Salmonella enterica subsp. *enterica* serovar 4,[5],12:i:- str. CVM23701	SeI_A4349	ZP_02570982.1	Conserved hypothetical protein	

Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2	STM0029	NP_459034.1	Putative transcriptional regulator	McClelland et al., 2001
Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2	STM0029	AAL18993.1	Putative transcriptional regulator	

[a] List of *Salmonella* spp, which have the identical STM0029 protein (identical sequence) was released based on the NCBI BLAST algorithm.

5. DISCUSSION

5.1. A putative *Salmonella* transcriptional regulator, STM0029, contributes to *Salmonella* intracellular survival through resistance against antimicrobial peptide killing

S. Typhimurium is one of the leading causes of human acute food-borne diseases worldwide. The key virulence factors that enable *Salmonella* to elicit pathogenesis are its ability to not only infect epithelial cells, but also survive within macrophages, which possess innate immune defense mechanisms against bacterial pathogens. To successfully proliferate within macrophages, *Salmonella* requires numerous virulence factors encoded within *Salmonella* Pathogenicity Islands (SPIs) and two component system regulators (TCSs) for expression and coordination of multiple genes involved in resistance against host cell defense mechanisms and acquisition of nutrients within the intracellular niche. In addition to currently known genes, new virulence determinants are being continuously reported from studies of host-microbes interactions in different animal models. In the present work, I have identified and characterised a putative transcriptional regulator STM0029 in *S.* Typhimurium SL1344 that contributes to *Salmonella* intracellular survival by affecting resistance to host antimicrobial peptide (AMP) killing.

Based on a previous study in our laboratory, we found that the expression of the *Salmonella* putative transcriptional regulator STM0029 is strongly reduced in a ppGpp-deficient strain of *Salmonella* (Δ*relA*Δ*spoT*) (Thompson et al., 2006). A ppGpp-deficient strain is unable to invade host epithelial cells or survive within macrophages. Because ppGpp affects only the distribution of free RNA polymerase between ribosomal RNA (rRNA) and messenger RNA (mRNA) promoters, promoters responsive to particular growth or environmental conditions remain inactive in response to changes in growth conditions. We have used this characteristic of ppGpp-deficient strains to identify genes, which show loss or reduced expression (relative to the wildtype strain) under conditions favoring virulence gene expression in *Salmonella* Typhimurium (Thompson et al., 2006). The previously uncharacterised open reading frame STM0029 was identified in this manner, and we hypothesized that the biological function(s) of STM0029 might be involved in either *Salmonella* invasion and/or intracellular survival.

Because of lack of reproducibility of host cell invasion assay results in 2008 – 2010 and 2011 (Table III), I also repeated antimicrobial peptide (AMP) killing assays to determine whether the phenotypes obtained for the ΔSTM0029 and Δ*pmrA/B* strains remained present regarding bactericidal peptide challenges in order to confirm the original observations.

Phenotypes (killing patterns) obtained from both strains remained consistent with prior results (shown in Fig. 6 and Fig. 7 for LL-37 and polymyxin B challenges), demonstrating that the experimental strains and their phenotypes against AMP challenges were correct. At present, I speculate that the different phenotypes regarding cell invasion assays might be due to serum effects, which have altered the cell physiology of the cell lines. Since both the ΔSTM0029 and the Δ*oafA* strains exhibited sensitivity against human and mouse sera challenges, it should be interesting to take a detailed look at serum effects on both pathogens and host cells in the future.

Bactericidal compounds, *e.g*. AMPs, nitric oxide and oxygen radicals have been reported to play crucial roles in human innate host defense for killing of a wide variety of bacterial pathogens. Most of these antimicrobial peptides are cationic in nature, and interact with the bacterial cytoplasmic membrane, composed of negatively charged phospholipids (Eswarappa et al., 2008). Increasing numbers of studies have demonstrated numerous novel genes involved in *Salmonella* defense against host AMP killing mechanisms. Detweiler et al. (2003) reported that VirK, SomA and RcsC are important for *S*. Typhimurium systemic infection and resistance to cationic peptides. The *yejABEF* operon in *Salmonella* has also been reported to be involved in resistance to AMP killing and contribution to virulence, although the mechanism is still unknown (Eswarappa et al., 2008). Defensins and cathelicidins (a human cathelicidin is known as LL-37) are mainly expressed and stored in mast cells and macrophages (Nijnik et al., 2009; Ouellette et al., 2001). The AMP killing assay in this study revealed that the ΔSTM0029 strain exhibited higher sensitivity to killing by human α-defensin-1, β-defensin-1, β-defensin-2 (Fig. 5) and LL-37 (Fig. 6) compared to the wildtype. In addition to STM0029 investigated in this study, PgtE, which encodes outer membrane protease in *S*. Typhimurium has also been reported to contribute to resistance against LL-37 and cathelicidin-related antimicrobial peptide (CRAMP) (mouse) challenges (Guina et al., 2000). LL-37 is of particular interest because it has a broad antimicrobial spectrum and displayed high activities [minimum inhibitory concentration (MIC) /10 μg/ml] against both Gram-positive and Gram-negative bacteria, *e.g*. *Pseudomonas aeruginosa*, *E. coli*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* (Turner et al., 1998). Rosenberger et al. (2004) reported that the murine cationic AMP cathelicidin-related CRAMP impairs *Salmonella* intracellular replication as well. Moreover, LL-37 exhibits different chemo-physical characteristics compared to those of native α-helical antimicrobial peptides isolated from invertebrates or vertebrates (Oren et al., 1999). Most important and unique characteristics of LL-37 are: (I) LL-37 exists in the form of

oligomers in solution; (II) LL-37 is well protected from proteolytic degradation; and (III) LL-37 recognizes both zwitterionic and negatively charged phospholipid vesicles as its targets.

I also determined the sensitivity of the ΔSTM0029 strain against one Gram-positive bacterium *Bacillus polymyxa*-produced antimicrobial peptide, polymyxin B (Fig. 7), since polymyxin has been reported to be mainly controlled by the *Salmonella* PmrA/B TCS (Mäkelä et al., 1978; Roland et al., 1993). In recent years, polymyxin B has re-emerged in medical practice for therapies (Evans et al., 2009; Falagas and Kasiakou, 2005) and its application will likely continue to increase, since few new drugs for the treatment of infections caused by multidrug-resistant (MDR) Gram-negative bacteria remain (Zavascki et al., 2007). In outbreaks caused by *Salmonella* spp, MDR strains have been reported for both *S*. Typhi (reviewed by Rowe et al., 1997) and *S*. Typhimurium (reviewed by Alcaine et al., 2007). However, although polymyxin B is one of the older classes of cationic peptide antibiotics, a substantial gap still exists in the knowledge of polymyxin B in pharmacology and its application (Mogi and Kita, 2009). To date, there are still no well-designed clinical trials to evaluate the efficacy of polymyxin B against infections caused by MDR Gram-negative bacteria (Zavascki et al., 2007). Based on the discussion from the reference reported by Zavascki et al. (2007), only three studies investigating the use of polymyxin B for treatment of MDR Gram-negative bacilli, mostly *Acinetobacter* spp and *Pseudomonas aeruginosa* are found (Holloway et al., 2006; Ouderkirk et al., 2003; Sobieszczyk et al., 2004). Regarding the mechanism of resistance by *Salmonella* against polymyxin B, the PmrA/B TCS (Winfield and Groisman, 2004) and the gene *mig-14* (Brodsky et al., 2002) have been reported to be involved. Based on the findings in this study, I found that loss of STM0029 led to reduced resistance of *Salmonella* against AMP challenges, including polymyxin B, LL-37 and defensins, which suggests that STM0029 is also critical for *Salmonella* resistance aginst not only natural AMPs in host cells, but also antibiotics in clinical treatment.

5.2. Expression of STM0029 is repressed by the PmrA/B two component system, and is PhoP/Q-independent

Similar to other bacterial pathogens, *Salmonella* requires numerous two component system (TCS) regulators for expression and coordination of multiple genes involved in virulence and pathogenesis. Currently, at least 38 TCSs have been found in *S*. Typhimurium, two of which, the PhoP/Q TCS and the PmrA/B TCS, have been well-studied and reported to be involved in *Salmonella* intracellular survival (Groisman and Saler, 1990; reviewed by Gunn, 2008; Miller

and Mekalanos, 1990; Miller, 1991). The *Salmonella* PhoP/Q TCS has been demonstrated to regulate hundreds of genes encoding the majority of virulence properties including intracellular survival, invasion, lipid A structure, resistance to AMPs, and phagosome alteration (Prost and Miller, 2008). Cheng et al. (2009) reported that the PhoP/Q TCS is also important for *Klebsiella* pathogenesis in addition to *Salmonella*. To date, more than 200 PhoP/Q-dependent genes are considered to be involved in different aspects of virulences by microarray analyses and motifs sceening (Monsieurs et al., 2005). The expression of STM0029 during the *in vitro* culture was not affected by loss of the PhoP response regulator in either L-broth or M9 minimal medium (Fig. 8A-F); however, the expression of STM0029 was sharply increased in a PmrA/B-deletion background (Fig. 8G-I). In addition, the expression levels of PhoP/Q-dependent genes including *pagP* (Ahn et al., 2004; Guo et al., 1998), *pagD* (Belden and Miller, 1994; Gunn et al., 1995), *pagC* (Miller, 1989; Pulkkinen and Miller, 1991) and *pcgL* (Hilbert et al., 1999) were not affected in the ΔSTM0029 strain compared to the wildtype, Δ*phoP* and Δ*pmrA*/*B* strains although the expression level of *pagD* appeared to be quite low compared to other genes (Fig. 9). These results indicate that STM0029 is not associated with the regulation of the expression of at least these four important PhoP-dependent genes within the PhoP/Q regulon. STM0029 is more likely present in a separate regulatory system. Moreover, results obtained from the β-galactosidase assays indicated that the PmrA/B TCS repressed the expression of STM0029; however, whether STM0029 directly or indirectly interacts with the PmrA/B TCS awaits further investigation.

At present, many *Salmonella* virulence factors involved in *Salmonella* intracellular survival and resistance to AMP challenges have been reported to be regulated by the PhoP/Q and the PmrA/B regulon (Groisman and Saler, 1990; Miller et al., 1989; Pulkkinen and Miller, 1991). In addition to these two well-studied two component systems, other *Salmonella* virulence factors, including *Salmonella* Pathogenicity Island-2 (SPI2) and *Salmonella* plasmid-encoded virulence factors, *e.g.* SpvB, SpvC and Rck have been reported to control critical regulatory systems involved in *Salmonella* pathogenesis as well (Hensel. et al., 2004; Matsui et al., 2001). However, the regulatory system(s) in which STM0029 is involved requires further investigation.

5.3. STM0029 is involved in the regulation of the genes involved in LPS O-antigen biosynthesis and modifications

In order to identify genes whose expressions might be affected/regulated by the putative transcriptional regulator encoded by STM0029, I performed microarray analyses and

compared whole genome expression profile obtained from the wildtype and the ΔSTM0029 strains, respectively. In addition to L-broth, which is generally used for generation of highly invasive *Salmonella* cultures, I also grew bacteria in PCN defined medium with limiting phosphate (0.4 mM Pi) to mimic the *Salmonella* intracellular milieu for total RNA preparations. PCN defined medium has previously been shown to result in activation of genes involved in intracellular growth and/or survival (Gerlach et al., 2007). I selected 20 PhoP/Q-regulated genes and 50 PmrA/B-regulated genes based on references from Groisman, E.A. (2001) and Marchal et al. (2004), respectively (Fig. 10; Supplementary Table II, see page 137-141) and compared mRNA expression levels between the ΔSTM0029 strain and the wildtype. In L-broth, mRNA expressions obtained from *phoQ* (STM1230), *pmrF* (STM2298), *pgtE* (STM2396), *prgJ* (STM2872) and *mgtC* (STM3764) showed slight increases. In contrast, expression from *ugtL* (STM1601) showed a slight decrese and expressions of the *prgI* (STM2873) and *pmrB* (STM4291) genes released 1.3- and 2-fold decreases, respectively. In PCN medium, the expression level of *pagA* (STM2080) showed a slight decrease and expression of *prgK* (STM2871), *prgJ* and *prgI* showed increases (Fig. 10). The *pagA* (*ugd*) gene, which encodes UDP-glucose-6-dehydrogenase is involved in the LPS lipid A modification (Morona et al., 1995). According to the observation in this study, expression of *pagA* was affected by loss of STM0029 in PCN defined medium; however, LPS patterns (bacterial LPS profile) obtained from bacteria grown in PCN medium showed no difference (data not shown). Moreover, although expression of *stfA* (STM0195, major fimbrial subunit) was affect by loss of STM0029, no significant difference of motility between the wildtype and the ΔSTM0029 strain was found either (Motility assays; data not shown). Diverse gene expression patterns might be due to different media compositions between L-broth and PCN medium. Compositions of L-broth are more complicated than PCN defined medium. Therefore, the greater numbers of genes were found by loss of STM0029 in L-broth compared to those were found in PCN medium might be due to other signals in L-broth. Expression of *hilA*, which is one major regulator of *Salmonella* SPI1 genes was not affected by loss of STM0029 in either media. This phenomenon is consistent with the observation that STM0029 is not involved in the process of *Salmonella* invasion (data not shown).

Gunn et al. (2008) reported that the contribution of the PmrA/B TCS to the resistance to polymyxin B challenge is through the regulation of genes involved in LPS lipid A modifications. Interestingly, genes affected by loss of STM0029 in this study were mainly involved in LPS O-antigen biosynthesis and modifications (Fig. 10; Table VI). This phenomenon could explain the observation that the ΔSTM0029 strain showed higher

sensitivity against various AMP challenges. Expression of the *wcaF* gene (STM2110), whose gene product functions in extracellular polysaccharide colonic acid synthesis showed a reduction by loss of STM0029 in PCN medium. Colonic acid has been reported to be important for *E. coli* to survive outside host cells and perhaps has a role involved in the resistance to desiccation (Ophir et al., 1994). The predicted function of WcaE, which encodes glycosyl transferase is involved in O-antigen biosynthesis. In addition, Mao et al. (2001) reported that WcaE also contributes to acid and heat tolerance in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7.

Slauch et al. (1995) reported that acetylation of O-antigen by OafA affects the three-dimensional structure of LPS and thus is involved in alterations in antigenic determinants. The function of OafA has been characterized. It belongs to a member of a family of integral membrane trans-acylases (Slauch et al., 1996) and determines the *Salmonella* O5-antigen (Slauch et al., 1995; Hauser et al., 2011). However, what genes and/or what regulatory systems in *Salmonella* control the function of OafA are still not clear. In this study, I showed that expression of *oafA* is specifically regulated by STM0029, since expression of *oafA* showed a decrease by loss of STM0029 instead of by loss of the PmrA/B TCS (Fig. 12). Moreover, the ΔSTM0029 strain displayed a similar phenotype against sera challenges as previously reported for an *oafA* deletion mutant in *Heamophilus influenzae* (Fig. 13; Fox et al., 2005), which suggests that STM0029 may be involved in the regulation of genes involved in post-translational modifications of *Salmonella* LPS required for resistance to host defense mechanisms. However, I was not yet able to detect a direct binding between the *oafA* promoter region and the purified STM0029 protein in electrophoretic mobility shift assays (EMSA; data not shown), indicating that other factors may be involved in regulation together with STM0029 and OafA.

Rfe has been reported to be required for the O:54 polysaccharide synthesis in *Salmonella enterica serovar* Borreze ($rfb_{O:54}$) (Keenleyside et al., 1994). The *rfe* gene product functions to transfer UDP-GlcNAc-undecaprenyl Phosphate N-acetylglucosaminyl 1-phosphate (Glc*p*NAc-1-P) to the carrier lipid in order to form the first lipid intermediated (lipid I) (Schnaitman and Klena, 1993). Lipid I, so called GluNAc-pyrophosphorylundecaprenol, is the product involved in the initial step to synthesize LPS O-antigen repeat units. Once lipid I is completed, lipid II and lipid III are allowed to attach on LPS O-antigen. The *wec* genes, including *wecB* (STM3920) and *wecC* (STM3921) are also identified from our microarray analysis. Functions of WecB and WecC are required for the synthesis of lipid III (Schnaitman and Klena, 1993). Additionally, Ramos-Morales et al.

(2003) reported that a mutation of *wecD* in *Salmonella* causes higher sensitivity against deoxycholate (DOC).

RfaH, which encodes a transcriptional antiterminator is involved in the biosynthesis of *S*. Typhimurium LPS O-antigen and LPS core region (Kong et al., 2009). In the absence of RfaH, *Salmonella* is unable to synthesize LPS O-antigen and LPS core region sugars. Quantitative RT-PCR results also confirmed that expression level of *rfaH*, which was reduced by loss of STM0029 (Fig. 11). A recent study has reported that RfaH also regulates the *wzy* gene, which is involved in LPS O-antigen repeat unit polymerization (Bravo et al., 2008). Wzx, Wxy and Wzz are responsible for LPS O-antigen assembly and export (Samuel and Reeves, 2003). WzzE, which encodes the enterobacterial commen antigen (ECA) polysaccharide chain length modulation protein, has also been released in my microarray analysis. Batchelor et al. (1991) first reported the function of Wzz involved in the determination of the legth of LPS O-antigen chain. Later, the *wzz* gene has been named wzz_{ST} (also called *cld* or *rol*) in order to distinguish it from another *wzz* gene homologue, wzz_{fepE}, which shares homology with *Escherichia coli fepE* (Murray et al., 2003). wzz_{ST} and wzz_{fepE} are two major genes in *Salmonella* involved in the determination of the the length of LPS O-antigen. The production of the LPS O-antigen modal banding pattern mediated by Wzz_{ST} is around 16-35 subunits (are called Long O-antigen; L-O-antigen) (Morona et al., 1995); in contrast, Wzz_{fepE} is responsible for the very long modal banding pattern, which contains over 100 subunits (are called Very-long O-antigen; VL-O-antigen) (Murray et al., 2003). A recent study has demonstrated that the production of O-antigen by *S*. Typhimurium SL1344 strain and *S*. Typhi are under the control of the bacterial growth phase: increased amounts of L-O-antigen, which are produced by *Salmonella* grown to the stationary phase confer a higher ability to resist human serum (Bravo et al., 2008). Moreover, *Shigella flexneri* 2a has also been demonstrated to show a differential, growth-phase regulation of O-antigen production (Carter et al., 2007).

Complement is one of the major bactericidal innate immune defense mechanisms in host cells (Dunkelberger and Song, 2010). *Salmonella* has been reported to be able to resist host complement in previous studies (Heffernan et al., 1992; Hölzer et al., 2009; Murray et al., 2006). To date, the mechanisms utilised by *Salmonella* for the resistance to host complement killing include shedding of C5b-9 from the cell surface (Joiner et al., 1982), inhibition of classical pathway activation *via* C1q/C1s binding molecules (Stemmer and Loos, 1985) and prevention of C9 polymerisation (Heffernan et al., 1992). Clay et al. (2008) reported that *Francisella tularensis* LPS O-antigen is also involved in evasion of

complement-mediated lysis. In this work, I found that the ΔSTM0029 strain showed higher sensitivities against human and mouse sera challenges (Fig. 13), which suggests that STM20029 should be required for *Salmonella* defense host innate immune system. However, the mechanism in which STM0029 involved to interfere with the function of the host complement system still needs to be investigated.

A number of genes involved in peptidoglycan (PG) biosynthesis were found to be affected in the ΔSTM0029 mutant in this study as well, including *lppB* (STM1376) (Sha et al. 2004) and *mrcA* (STM3493) (Meberg et al., 2001). Braun (murein) lipoprotein (Lpp) is also one of the major components used to establish the outer membrane of Gram-negative enteric bacteria in addition to LPS. Lpp functions as a potent stimulator of inflammatory and immune responses. Functions of LppB, which is a functional copy of Lpp, has been demonstrated by Sha et al. (2004). LppB causes not only an increase of the expression level of TNF-α and IL-6, but also greater tissue damage in the liver and the spleen (Fadl et al., 2005). The *mrcA* (STM3493) gene product, which has a predicted function of bifunctional murein transglycosylase/murein transpeptidase, is involved in the synthesis of cross-linked peptidoglycan from the lipid intermediates during the cell wall formation.

5.4. Current model and perspectives

Based on the results observed in this study, I propose a model in which STM0029 acts as a regulator involved in the expression of genes required for *Salmonella* intracellular survival within host cells (Fig. 15; see page 96). Although the signal(s) involved in the expression of STM0029 remain unknown, I believe that the signal(s) could be involved in many aspects of changes in environmental conditions (intracellular niches), *e.g.* acid, concentration of intracellular ions and/or host bactericidal compounds. As shown in Fig. 15B, once the activity of STM0029 is triggered, the expression of gene products involved in LPS O-antigen biosynthesis and modifications are further directly and/or indirectly activated by STM0029, contributing to *Salmonella* resistance against host antimicrobial peptides challenges and to survival within the unfavourable intracellular niche.

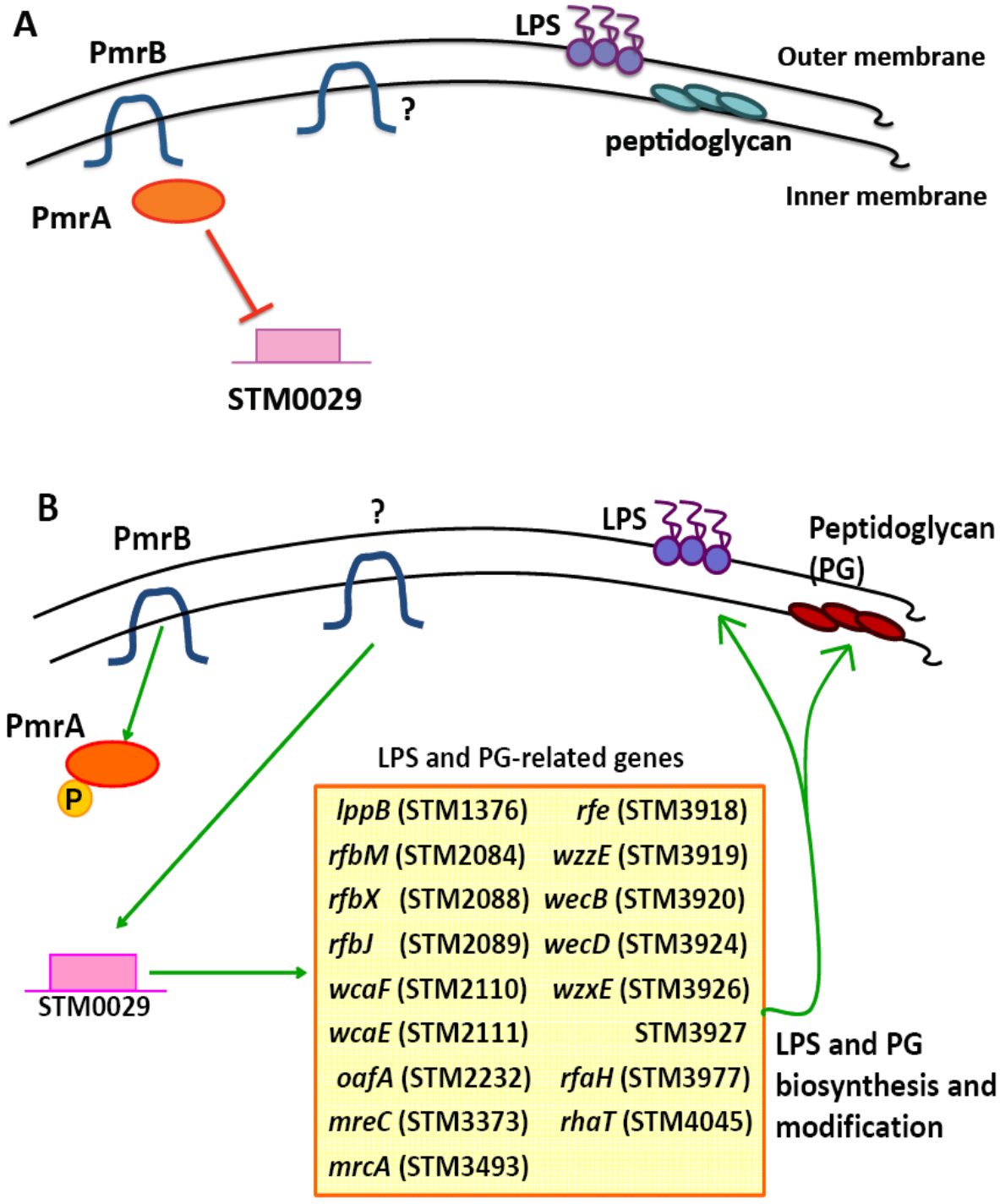

Fig. 15. Current model for the possible role of STM0029 in regulation of genes involved in LPS O-antigen biosynthesis and modifications. (A) Before the uptake by host cells, expression of STM0029 is repressed by the *pmrA/B* two component system. (B) Once *Salmonella* infects host cells, expression of STM0029 is activated. Therefore, the STM0029 gene product is able to control expressions of downstream genes involved in LPS O-Antigen biosynthesis and modifications. Red blunted lines represent the repression of target genes and green lines with arrows represent the activation of target gene.

In the present investigation I have shown that expression of STM0029 is repressed by the PmrA/B TCS; in the absence of the PmrA/B TCS, expression of STM0029 is strongly activated. However, it is remains unclear whether STM0029 is a part of the PmrA/B regulon or it acts as a connector and/or coactivator, which functions in crosstalk with different regulatory networks. Based on my microarray analyses (Fig. 12), 730 genes are co-regulated by STM0029 and the PmrA/B regulon (15.86 % of total genes), implying that STM0029 should be somehow strongly associated with the PmrA/B regulatory system. Furthermore, *oafA* was identified in this study as a specific STM0029-regulated gene. Fox et al. (2005) reported that OafA-encoded acetylation is essential for *Haemophilus influenzae* resistance to human serum killing; however, the regulatory system(s) and regulators involved in control of this mechanism in *H. influenzae* are also unknown. In this study, I have shown that STM0029 contributes to both aspects of the LPS modifications and the resistance to host sera, which suggests that STM0029 may function to link both aspects.

To test this hypothesis, a plasmid-encoded *oafA* will be introduced into the ΔSTM0029 strain and investigate whether this experimental strain is capable to compensate for a defect against AMP challenges. Moreover, a plasmid-encoded STM0029 will be transducted into strains harboring transcriptional *lacZ* fusions with STM0029 to elucidate whether STM0029 serves as an autoregulator. Additionally, *in vitro* and/or *in vivo* signals involved in activation of expression of STM0029 should be further investigated.

In this study, the function of STM0029 has been shown to contribute to *Salmonella* resistance against AMP challenges. Antimicrobial peptides are one of the major mechanisms to eliminate invasive pathogens within host cells, which implys that alterations of environmental conditions and/or *Salmonella* intracellular niche (*Salmonella*-containing vacuole; SCV) are possible signal sources, which are able to stimulate expression of STM0029, *e.g.* acidification of *Salmonella*-containing vacuole, the alteration of intracellular ionic concentrations and encountering host antimicrobial peptides and host complement.

In addition to genes involved in LPS O-antigen biosynthesis and modifications, the roles of other possible STM0029-regulated genes which are not involved in *Salmonella* virulence and the LPS/bacterial cell wall biosynthesis are of interest. Based on the results shown in Fig. 12, expression of numerous genes involved in the category of “carbohydrate transport and metabolism” (8.0%; light blue), “post-translational modification, protein turnover and chaperones” (7.1%; brown) and “intracellular trafficking, secretion and vesicular transport” (6.3%; light purple) are affected by loss of STM0029 as well. In the future, Chromatin Immunoprecipitation-on-Chip (ChIP-on-chip) methods and deep sequencing might

be carried out to aid to identify possible candidate genes, which directly interact with STM0029 in order to provide us a more comprehensive picture of the STM0029-regulated network.

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7. SUPPLEMENT

Supplement I. Laboratory equipments and materials

Laboratory equipments	
Agarose gel electrophoresis tank	B1A and GE-B2, AGS, Heidelberg
Agilent 2100 Bioanalyzer	Agilent Technologies, UK
Aspirator	Vacuboy, neolab, heidelberg
Autoclave	GTA16, Gössner, Hamburg
Axon GenePix 4000AScanner	Axon Instruments, USA
Centrifuges	Rotina 46R, Hettich Zentrifugen, Tuttlingen 3K30, Sigma Laborzentrifugen, Osterode 5415D, eppendorf Zentrifugen, Engelsdorf Avanti J-25, Beckmann Coulter, Krefeld
Contrast microscope	Telaval 31, Zeiss, Jena
Nanodrop	peqLab, Erlangen
Electronic balance	BL3100, Sartorius, Göttingen
Electronic precision weighing instrument	BP210S, Sartorius, Göttingen
Electroporator	EasyjecT Prima, peqLab, Erlangen
Electrophoresis power supply	PS 250, Hybaid, Lexington, USA
Freezer -20	Liebherr comfort GS1313, Ochsenhausen
Freezer -70	Advantage, Nunc, Wiesbaden
Heating block	Thermomixer compact, Eppendorf, Hamburg
Hybridisation chambers	Anachem, UK
Image-Dokumentation instrument	UV-Transilluminator UVT 28 MP, Camera E.A.S.Y. 429 K, Documentationssystem RM6, Herolab, Wiesloch
Image-Dokumentation photo printer Incubators	P93E, Mitsubishi Electric, Ratigen CB150, Binder, Tuttlingen VT 5042 EK/N2, Heraeus, Hanau
LABMATE Precision pipettes	ABIMED GmbH, Langenfeld
Magnetic stirrer	IKWAG RET, Th. Karow, Berlin
pH-Meter	Mikroprozessor pH-Meter 740, Knick, Berlin
Pipetting aid	Pipetus-akku, Hirschmann, Eberstadt
Refrigerator	Privileg 4873, Quelle, Fürth
Scanner	DUOSCAN T1200, AGFA, Köln
SDS-PAGE-apparatus	Biorad, USA
Shaking incubator	GFL3031, GFL, Burgwedel
Spectrophotometer	Ultrospec 3000 pro, Amerscham, Freiburg
Sterile work bench	LaminAir HB 2448 and LB-48-C, Heraeus, Hanau
Stratalinker	Stratagene, UK
Thermocycler	T3 Thermocycler, Biometra, Göttingen
Vaccum pump	N735 AN18, KNF Neuberger, Freiburg
Vortexer	MS2 Minishaker, IKA, Staufen
Waterbath	Grant OLS 200, CLF Laborgeräte, Burgwedel E-5, Julabo, Seelbach
Consumables	
Amicon ultra centrifugal filters	Millipore, Germany
Cell culture flasks	Corning, Schiphol-Rijk, Netherland
Cell scraper	Corning, Schiphol-Rijk, Netherland
Nunc Cryotubes	Laborversand, Würzburg
Disposable cuvettes	MBT, Giessen
Disposable pipettes	Costar, Bodenheim
Electroporation cuvettes	2 mm, peqLab, Erlangen

Filter paper	Whatmanpaper 3MM, Schleicher and Schüll, Dassel
Pasteur pipettes	Roth, Karlsruhe
Petriplates	Sarstedt, Nürnbrecht
Sterile filters	Rotilabo-Spritzzenfilter 0.45 µM and 0.22 µµ pore size, Roth, Karlsruhe
12-well cell culture plates	CellBIND, Corning, Schiphol-Rijk, Netherland

Reagents

Acetic acid	Roth, Karlsruhe
Acrylamide-Bisacrylamide-Solution, Rotiphorese Gel 30	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Alizarin yellow	Sigma-Aldrich, München
Ammoniumpersulfate (APS)	Sigma-Aldrich, München
Anhydrous 1,2-dichloroethane (DCE)	Sigma-Aldrich, UK
Anhydrous succinic anhydride	Sigma-Aldrich, UK
Antibiotics	Sigma-Aldrich, München
L-Arabinose	Roth, Karlsruhe
Boric acid	Roth, Karlsruhe
Bovine serum albumin (BSA)	Sigma-Aldrich, München
Bromophenol blue	Serva, Heidelberg
5-Bromo-4-chloro-3-indolyl-D-galactoside (X-Gal)	Sigma-Aldrich, München
Calcium chloride	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Coomassie brilliant blue R250	Serva, Heidelberg
Cy3	GE Healthcare Lifesciences, UK
Cy5	GE Healthcare Lifesciences, UK
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, München
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe
Dithiothreitol (DTT)	Sigma-Aldrich, München
DMEM/Ham‘s F-12 salts medium	Biochrom, Berlin
dNTPs	Roth, Karlsruhe
Ethanol pure	Merck, Darmstadt
Ethylenedinitrotetraacetic acid (EDTA)	Roth, Karlsruhe
Ethylene-glycol-bis-tetraacetic acid (EGTA)	Roth, Karlsruhe
Fetal calf serum	PAN Biotech, Germany
Gentamycin	Biochrom, Berlin
Glucose	Sigma-Aldrich, München
Glycerin	Roth, Karlsruhe
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	Roth, Karlsruhe
Magnesium chloride	Roth, Karlsruhe
Magnesium sulphate	Roth, Karlsruhe
Methanol	Roth, Karlsruhe
n-Methylimidazole	Sigma-Aldrich, UK
o-Nitrophenyl-ß-D-galactopyranoside (ONPG)	Sigma-Aldrich, München
PBS	Biochrom, Berlin
Phenol	Roth, Karlsruhe
Phenylmethylsulfonylfluoride (PMSF)	Roth, Karlsruhe
Potassium chloride	Roth, Karlsruhe
Random hexamers	Invitrogen, UK
Sodium carbonate	Merck, Darmstadt
Sodium chloride	Roth, Karlsruhe

Sodium citrate	Sigma-Aldrich, München
Sodium dihydrogen phosphate	Roth, Karlsruhe
di-Sodium hydrogen phosphate	Roth, Karlsruhe
Sodiumdodecyl sulfate (SDS)	Roth, Karlsruhe
TEMED	Biorad, USA
Tris Base	Sigma-Aldrich, München
Triton X-100	Sigma-Aldrich, München
Trypsin-EDTA	Biochrom, Berlin
Tryptone	Roth, Karlsruhe
Ultra-pure water	Sigma-Aldrich, UK
Wasserblau / Anilin blue	Fluka-Riedel-de Haen, Seelze
Xylene cyanol	Fluka-Riedel-de Haen, Seelze
Yeast extract	Roth, Karlsruhe
Commercial Kits	
Abgene Extensor Hi-Fidelity PCR master mix	Thermo Scientific, UK
BCA Protein Assay Kit	Pierce, USA
BioPrime DNA labeling system	Invitrogen, UK
100 bp-DNA Ladder	Roth, Karlsruhe
Lambda HindIII marker	Roth, Karlsruhe
Protein molecular weight marker Vll-L	Sigma-Aldrich, München
Protein Ni-TED column	Machery-Nagel, Düren
QIAGEN Plasmid Midi Kit	Qiagen, Hilden
QIAGEN Genomic DNA preparation kit	Qiagen, Hilden
Qia-quick PCR purification kit	Qiagen, UK
Enzymes	
Affinity Script multi-temperature Reverse Transcriptase	Stratagene, UK
Bio Therm Taq-Polymerase	Rapidozym, Berlin
DNA-Restriction enzymes	Promega, Mannheim
Lysozyme	Roth, Karlsruhe
RNase free DNase	Promega, UK
T4 DNA-Ligase	Promega, Mannheim
Solutions, Media and Buffers	
Agarose gel electrophoresis buffers	
6 x Agarose-Loading buffer	40% Sucrose 0.25% Bromphenolblue 0.25% Xylene cyanol
Ethidiumbromide solution	1% in sterile H_2O
10X TBE-buffer	890 mM Tris-base 890 mM Boric acid 20 mM EDTA
Bacterial culture medium	
IPTG stock solution	0.1 M IPTG in dest. H_2O
L-broth	0.5% NaCl 1% Trypton 0.5% Yeast extract in H_2O, autoclaved
1X M9 minimal medium	50.0 ml 10X M9 salts 5.0 ml 20 % Glucose

	0.5 ml 1 M $MgSO_4$ 0.5 ml 10 mg/ml Ca^{2+}-Pantothenate 0.5 ml 10 mg/ml Thiamine 0.5 M $CaCl_2$ in 445ml of H_2O, autoclaved
PCN defined medium	100 ml 10 X MOPS Mixture 10 ml 0.132 M K_2HPO_4 in 580 ml sterile H_2O, filter
LB agar	L-broth 1.5% Agar, autoclaved
Top Agar	L-broth 0.7% Agar, autoclaved
X-Gal	25 mg/ml in Dimethylformamide in cultures: 50 µg/ml
Green plates	0.8% Tryptone 0.1% Yeast extract 0.5% NaCl 1.5% Agar, autoclaved 16.8 ml of 40% Glucose in 1000 ml 0.0625% Alizarin yellow 0.0067% Water bleu
Antibiotics	
Carbenicillin	10 mg/ml in H_2O In cultures: 100 µg/ml
Chloramphenicol	10 mg/ml in H_2O In cultures: 20 µg/ml
Kanamycin	10 mg/ml in H_2O In cultures: 50 µg/ml
Tetracyclin	10 mg/ml in 70 % Ethanol In cultures: 20 µg/ml
Beta-Galactosidase assay	
2X Z buffer	0.12 M $Na_2HPO_4 \cdot 7H_2O$ 0.08 M $NaH_2PO_4 \cdot H_2O$ 0.02 M KCl 0.002 M $MgSO_4 \cdot 7H_2O$ in 1000 ml ddH_2O, filter sterilzed
ß-mercaptoethanol is added immediately before the assay with a concentration of 0.1 M (stock: 14.3 - 14.4 M)	
RNA preparation	
Stop solution	95% absolute ethanol 5% phenol
Resuspension buffer	25 mM Tris (pH 7.0 -7.2) 1 mM EDTA

	in DEPC-treated water
Lysis solution	0.6 M NaOAc (pH ≈ 5.0) 4.0 mM EDTA 3.0% SDS
RNA resuspension buffer	0.1 mM EDTA
RT-PCR fomula	
ddH_2O	32.3 µl
10X reaction buffer	5.0 µl
dNTP	5.0 µl
$MgCl_2$	1.5 µl
Probe DANN	5.0 µl
Forward primer	2.0 µl
Reverse primer	2.0 µl
Taq polymerase	0.2 µl
PR-PCR program	
step 1	94ºC, 3 minutes
step 2	94ºC, 1 minute
step 3	50ºC, 1 minute
step 4	72ºC, 1 minute and repeat from step 2 for 34 cycles
step 5	72ºC, 10 minutes
step 6	4ºC, pause
Microarray atudies	
50X dNTPs	25 mM dATP 25 mM dGTP 25 mM dTTP 10 mM dCTP
10X dNTPs	1.2 mM each of dATP, dTTP, dGTP 0.6 mM dCTP 10 mM Tris pH 8.0 1 mM EDTA
RT reaction mix	2 µl of 10X RT buffer 2 µl of 0.1 M DTT 0.6 µl of 50X dNTPs
20X SSC buffer	3 M NaCl 0.3 M sodium citrate, pH 7.0
50X Denhardt‘s solution	1% Ficoll 1% polyvinylpyrrolidone
Hybridisation buffer	1.5 µl of 50X Denhardt‘s solution 2.25 µl of 20X SSC 1.125 µl of *E. coli* RNA (10µg/µl) 0.375 µl of 1 M HEPES, pH 7.0
Softwares	
Genespring microarray analysis software	silicon Genetics, UK
Sigma plot® 10	Systat software, USA

Supplement II. Relative transcript levels in Δ STM0029 compared to the wildtype[a] (rel. expression level [Δ STM0029/wildtype])[b]

No.	Gene	Predicted functions	Fold Change (log)		Reference
PhoP/Q-regulated genes[c]			**L-broth**	**PCN[d]**	
STM0628	*pagP*	Catalyzes the transfer of palmitate to lipid A	0.03	0.26	Bishop et al., 2000
STM1246	*pagC*	Outer membrane protein with sequence sililarity to *Enterobacter* OmpX	-0.02	-0.02	Gunn et al., 1995
STM1230	*phoQ*	Sensor protein PhoQ	0.38	0.08	Miller et al., 1989
STM1231	*phoP*	DNA-binding transcriptional regulator PhoP	-0.07	0.00	Fields et al., 1989
STM1465	*pcgL*	D-alanyl-D-alanine dipeptidase	0.17	-0.04	Hilbert et al., 1999
STM1601	*ugtL*	Putative membrane protein	-0.25	0.06	Shi et al., 2004
STM2080	*pagA*	UDP-D-glucose dehydrogenase	-0.14	-0.20	Morona et al., 1995
STM2298	*pmrF*	Synthesis and/or incorporation of α-aminoarabinose into lipid A	0.26	-0.10	Gunn et al., 1998
STM2304	*pmrD*	Polymyxin resistance protein B	-0.07	-0.10	Kox et al., 2000
STM2396	*pgtE*	Outer membrane protase; resistance to peptide C18G	0.24	0.10	Guina et al., 2000 Pietilä et al., 2005
STM2871	*prgK*	Needle complex inner membrane lipoprotein	0.05	0.31	Sukhan et al., 2003
STM2872	*prgJ*	Needle complex minor subunit	0.33	0.37	Sukhan et al., 2003
STM2873	*prgI*	Needle complex major subunit	-0.29	0.26	Sukhan et al., 2003
STM2874	*prgH*	Needle complex inner membrane protein	0.15	0.14	Sukhan et al., 2003
STM2876	*hilA*	Invasion protein regulator	0.10	0.10	Rodriguez et al., 2002
STM3763	*mgtB*	Pathogenicity island encoded protein: SPI3; Mg^{2+} transport ATPase, P-type 2	0.08	0.03	Snavely et al., 1989
STM3764	*mgtC*	Pathogenicity island encoded protein: SPI3; Mg^{2+} transport ATPase protein C	0.31	-0.13	Alix et al., 2003

STM4291	*pmrB*	*S*. Typhimurium sensor protein BASS/PMRB	-0.44	-0.14	Merighi et al., 2005
STM4292	*pmrA*	Putative RBS for BasR	-0.19	-0.15	Merighi et al., 2005
pSLT039	*spvB*	*Salmonella*-encoded virulence plasmid gene	0.18	-0.08	Fierer et al., 1993

[a] Protein encode by differentially expresssed genes were identified by annotation or homology to known proteins (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome) and assigned to categories based on description in clusters of orthologous groups of proteins (COGs).

[b] Genes, whose expressions showed the natural log of hybridization signal ratios (rel. expression level [Δ STM0029/wildtype]) ≥ 0.33 were selected.

[c] PhoP/Q-related genes are selected according to the reference from Groisman, E.A. (2001).

[d] PCN medium contains 0.4 mM Pi.

No.	Gene	Predicted functions	Fold Change (log)		Reference
PmrA/B-regulated genes[a]			**L-broth**	**PCN[b]**	
STM0115	*leuO*	Probable activator protein in leuabcd operon	0.27	-0.05	Hernández-Lucas et al., 2008
STM0195	*stfA*	Major fimbrial subunit StfA	-0.14	-0.44	Humphries et al., 2005
STM0275	*yafC*	Hypothetical protein; putative cytoplasmic protein	-0.06	-0.36	
STM0304	*sinR*	*S.* Typhimurium SINR protein	-0.16	0.09	Fredrick et al., 1996
STM0313	*dinP*	devoid of proofreading; Damage-inducible protein P; similar to *E. coli* damage-inducible protein P	0.02	0.19	
STM0605	*ybdN*	3'-phosphoadenosine 5'-phosphosulfate sulfotransferase	0.00	0.12	
STM0606	*ybdO*	Putative LysR family transcriptional regulator	0.08	0.14	
STM0708	*ybfA*	Putative periplasmic protein	0.05	0.16	
STM0732	*sdhC*	Succinate dehydrogenase, cytochrome b556	-0.28	-0.04	
STM0865	*ybjG*	Putative permease	-0.35	-0.03	
STM0987	*ycaR*	Ortholog of *E. coli* orf, hypothetical protein	0.19	0.52	
STM1161	*yceP*	Putative cytoplasmic protein	0.23	0.07	
STM1444	*slyA*	MarR family transcriptional regulator for hemolysin	0.01	-0.03	Song et al., 2008
STM1445	*slyB*	Outer membrane lipoprotein SLYB precursor	-0.18	-0.07	Song et al., 2008
STM1479	*pntA*	Pyridine nucleotide transhydrogenase, alpha subunit	-0.28	-0.08	
STM1631	*sseJ*	Regulated by SPI-2; secreted effector J SseJ (gi|9931614); *Salmonella* translocated effector	0.05	0.11	Freeman et al., 2003
STM1801	*ycgO*	Ortholog of *E. coli* orf, hypothetical protein	-0.17	0.10	

STM1852	*yebW*	Putative inner membrane lipoprotein	0.14	0.04	
STM1868A	*mig-3*	Phage tail assembly protein	0.06	0.08	
STM2080	*pagA (ugd/ pmrE)*	*S.* Typhimurium UDP-glucose 6-dehydrogenase	-0.14	-0.20	Morona et al., 1995
STM2119	*yegH*	Putative inner membrane protein	0.01	0.07	
STM2220	*yejG*	Putative RBS for *yejG*	-0.26	0.08	
STM2298	*pmrF*	Putative glycosyl transferase	0.26	-0.10	Gunn et al., 1998
STM2301	*pqaB*	PqaB; affects polymyxin B resistance and lipopolysaccharide synthesis; putative melittin resistance protein	0.01	-0.14	Baker et al., 1999
STM2304	*pmrD*	Polymyxin B resistance protein PMRD; polymyxin resistance protein B	0.25	-0.07	Perez et al., 2007
STM2395	*pgtE*	Phosphoglycerate transport; outer membrane protease E precursor	0.24	0.10	Pietilä et al., 2005
STM2396	*pgtA*	Phosphoglycerate transport; phosphoglycerate transport system transcriptional regulatory protein PGTA	-0.14	-0.05	Yang et al., 1988
STM2397	*pgtB*	Phosphoglycerate transport; phosphoglycerate transport system sensor protein PGTB	0.03	0.01	Niu et al., 1995 Yang et al., 1988
STM2398	*pgtC*	phosphoglycerate transport; Phosphoglycerate transport regulatory protein PGTC precursor	0.00	-0.17	Niu et al., 1995
STM2399	*pgtP*	Phosphoglycerate transport; phosphoglycerate transporter protein	-0.27	0.21	Niu et al., 1995
STM2400		Putative inner membrane protein	0.36	-0.25	
STM2826	*csrA*	Putative RBS for *csrA*	0.08	0.01	Altier et al., 2000

STM2828	*oraA*	Similar to *E. coli* regulator, OraA protein	0.15	0.01	
STM2982	*gcvA*	LysR family;regulator of *gcv* operon	-0.39	0.10	
STM3029	*stdA*	Similar to *E. coli* putative fimbrial-like protein	0.31	0.06	Chessa et al., 2008
STM3313	*yrbF*	Putative ABC superfamily transport protein	0.15	0.02	
STM3361	*yhcN*	Putative outer membrane protein	-0.35	0.15	
STM3538	*glgB*	1,4-alpha-glucan branching enzyme	-0.22	-0.03	Steiner and Preiss., 1977
STM3607	*yhjC*	Putative LysR family transcriptional regulator	0.12	0.16	
STM3640	*ipfA*	Long polar fimbria protein A precursor	0.26	0.08	
STM3707	*yibD*	Putative glycosyltransferase	-0.39	-0.16	Marchal et al., 2004 Merighi et al., 2005
STM3761	*slsA*	Pathogenicity island encoded protein: SPI3	0.23	0.25	
STM3869	*atpF*	Putative RBS for *atpF*	-0.06	0.08	
STM4118	*cptA*	Putative integral membrane protein	-0.14	-0.02	Tamayo et al., 2005
STM4291	*pmrB*	*S.* Typhimurium sensor protein BASS/PMRB	-0.44	-0.14	Merighi et al., 2005
STM4292	*pmrA*	Putative RBS for *basR*	-0.19	-0.15	Merighi et al., 2005
STM4293	*yjdB*	Putative RBS for *yjdB*	-0.35	0.13	
STM4294	*yjdE*	Putative APC family Putrescine/ornithine transport protein	0.06	0.04	
STM4470	*yjgD*	Hypothetical 15.7 dda protein in *argI-miaE* intergenic region	-0.04	-0.05	
STM4600	*lasT*	Putative RNA methyltransferase	0.15	-0.04	

[a] PmrA/B-related genes are selected according to the result obtained from Marchal et al. (2004).

[b] PCN medium contains 0.4 mM Pi.

No.	Gene	Predicted functions	Fold Change (log)		Reference
LPS, PG and cell wall-associated genes			**L-broth**	**PCN[a]**	
STM0048	*slpA*	Ortholog of *E. coli* probable FKBX-type 16KD peptidyl-prolyl cis-trans isomerase (a rotamase)	-0.30	-0.03	
STM0049	*lytB*	Similar to *E. coli* control of stringent response; involved in penicillin tolerance (AAC73140.1)	-0.25	0.03	Begley et al., 2002
STM0123	*murE*	UDP-N-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate ligase	-0.25	-0.12	
STM0124	*murF*	D-alanine:D-alanine-adding enzyme	-0.30	-0.04	
STM0125	*mraY*	Phospho-N-acetylmuramoyl-pentapeptide transferase	-0.18	0.03	
STM0126	*murD*	Ortholog of *E. coli* UDP-N-acetylmuramoylalanine-D-glutamate ligase	-0.02	-0.01	
STM0127	*ftsW*	Ortholog of *E. coli* cell division; membrane protein involved in shape determination	0.02	0.07	
STM0128	*murG*	UDP-N-acetylglucosamine:N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	-0.11	0.02	
STM0129	*murC*	L-alanine adding enzyme; UDP-N-acetyl-muramate:alanine ligase	0.22	0.04	
STM0130	*ddlB*	Ortholog of *E. coli* D-alanine-D-alanine ligase B, affects cell division	-0.20	-0.12	Zawadzke et al., 1991
STM0134	*lpxC*	UDP-3-O-acyl N-acetylglucosamine deacetylase	-0.70	-0.06	McClerren et al., 2005 Langklotz et al., 2010
STM0190	*mrcB*	Ortholog of *E. coli* peptidoglycan synthetase; penicillin-binding protein 1B	-0.48	-0.05	
STM0226	*lpxD*	Transfer of ß-hydroxymyristic acid from ACP	0.06	-0.01	
STM0228	*lpxA*	Transfer of ß-hydroxymyristic acid from ACP	-0.24	0.06	Anderson et al., 1987
STM0375	*ampH*	Penicillin- binding protein	-0.01	0.04	Gantois et al., 2006
STM0444	*ampG*	Muropeptide transporter	-0.21	0.05	Folkesson et al., 2005

STM0445	*yajG*	Putative lipoprotein	-0.51	0.02	
STM0446	*bolA*	Ortholog of *E. coli* possible regulator of murein genes	0.28	0.01	
STM0447	*tig*	Ortholog of *E. coli* trigger factor; a molecular chaperone involved in cell division	-0.45	-0.06	
STM0637	*dacA*	Ortholog of *E. coli* D-alanyl-D-alanine carboxypeptidase, fraction A; penicillin-binding protein 5	-0.13	0.17	Baquero et al., 1996
STM0638	*rlpA*	Putative RBS for *rlpA*	-0.21	0.07	
STM0639	*mrdB*	Putative RBS for *mrdB*	-0.36	0.08	
STM0640	*mrdA*	Similar to *E. coli* cell elongation, e phase; peptidoglycan synthetase; penicillin-binding protein 2	0.23	0.21	
STM0863	*dacC*	Ortholog of *E. coli* D-alanyl-D-alanine carboxypeptidase; penicillin-binding protein 6	-0.13	-0.11	Baquero et al., 1996
STM0988	*kdsB*	Synthesis of CMP-KDO from KDO-8-phosphate	0.00	0.00	
STM1376	*lppB*	Putative methyl-accepting chemotaxis protein	-0.38	-0.30	Fadl et al., 2005
STM1377	*lpp*	Murein lipoprotein	-0.07	-0.24	Fadl et al., 2005
STM1518	*marB*	Putative RBS for *marB*	0.37	-0.56	Randall et al., 2001
STM1519	*marA*	AraC/XylS family transcriptional activator of defense systems; multiple antibiotic resistance protein	0.02	-0.24	Randall et al., 2001
STM1520	*marR*	Multiple antibiotic resistance protein MARR	0.55	0.01	Randall et al., 2001; Sulavik et al., 1997
STM1521	*marC*	Multiple antibiotic resistance protein MARC	-0.11	-0.04	
STM1679	*mppA*	Periplasmic murein tripeptide transport protein	-0.22	-0.02	Park and Uehara, 2008
STM1772	*kdsA*	Synthesis of KDO-8-phosphate	0.06	0.00	
STM1845	*prc*	Tail-specific protease	-0.42	-0.07	Bäumler et al., 1994
STM2062	*dacD*	DD-carboxypeptidase	-0.02	-0.05	Baquero et al., 1996
STM2082	*rfbP*	Initiates synthesis of LPS O unit	-0.12	0.01	Jiang et al., 1991
STM2083	*rfbK*	Synthesis of GDP-rhamnose from mannose-1-phosphate	-0.22	0.09	Jiang et al., 1991
STM2084	*rfbM*	Synthesis of GDP-rhamnose from mannose-1-phosphate	-0.49	-0.10	Jiang et al., 1991
STM2085	*rfbN*	Synthesis of LPS O unit	-0.33	-0.06	Reeves, 1993
STM2086	*rfbU*	Synthesis of LPS O unit	-0.35	-0.01	Reeves, 1993
STM2087	*rfbV*	Synthesis of LPS O unit	-0.06	-0.08	Reeves, 1993
STM2088	*rfbX*	Assembly or transfer of LPS O unit	-0.49	-0.09	Reeves, 1993
STM2089	*rfbJ*	Synthesis of CDP-abequose	-0.45	-0.08	Reeves, 1993

STM2090	*rfbH*	Synthesis of CDP-4-keto-3,6,-Dideoxyglucose from CDP-glucose	-0.08	0.09	Reeves, 1993
STM2091	*rfbG*	Synthesis of CDP-4-keto-3,6,-dideoxyglucose from CDP-glucose	-0.09	0.11	Reeves, 1993
STM2092	*rfbF*	Synthesis of CDP-4-keto-3,6,-dideoxyglucose from CDP-glucose	-0.37	-0.06	Reeves, 1993
STM2093	*rfbI*	Synthesis of CDP-4-keto-3,6,-dideoxyglucose from CDP-glucose	-0.38	0.04	Reeves, 1993
STM2094	*rfbC*	Glucose-1-phosphate thymidylyltransferase	-0.39	-0.09	Jiang et al., 1991
STM2095	*rfbA*	Glucose-1-phosphate thymidylyltransferase	-0.40	0.01	Lindquist et al., 1993 Jiang et al., 1991
STM2096	*rfbD*	Glucose-1-phosphate thymidylyltransferase	-0.58	0.00	Jiang et al., 1991
STM2097	*rfbB*	Glucose-1-phosphate thymidylyltransferase	-0.03	0.07	Jiang et al., 1991
STM2107	*wcaH*	GDP-mannose mannosyl Hydrolase [*Salmonella* Typhimurium LT2].	-0.47	0.00	Domínguez-Bernal et al., 2004
STM2108	*wcaG*	Bifunctional GDP fucose synthetase	-0.74	-0.24	
STM2109	*gmd*	GDP-D-mannose dehydratase	-0.11	0.03	
STM2110	*wcaF*	Putative acyltransferase	0.08	-0.94	Ophir et al., 1994
STM2111	*wcaE*	Putative RBS for *wcaE*	0.07	-0.38	
STM2112	*wcaD*	Putative RBS for *wcaD*	0.65	0.01	
STM2113	*wcaC*	Putative glycosyl transferase	0.06	-0.32	
STM2114	*wcaB*	Putative acyl transferase	-0.09	0.03	
STM2115	*wcaA*	Putative glycosyl transferase	-0.68	0.13	
STM2116	*wzc*	Putative RBS for *wzc*	0.10	0.16	
STM2117	*wzb*	Putative protein-tyrosine-phosphatase	0.03	-0.07	
STM2118	*wza*	Putative polysaccharide export protein	0.14	0.14	Wehland et al., 2000
STM2188	*mglC*	Putative RBS for *mglC*	-0.28	-0.05	Müller et al., 1985
STM2189	*mglA*	Putative RBS for *mglA*	-0.07	-0.10	Müller et al., 1985
STM2190	*mglB*	Galactose transport protein	0.09	-0.05	Müller et al., 1985
STM2191	*galS*	GalR/LacI family Transcriptional repressor of *mgl* operon	0.27	-0.06	Yoshimura et al., 2006
STM2232	*oafA*	O-antigen five; acetylation of the O-antigen (LPS)	-0.20	-0.21	Slauch et al., 1995 Slauch et al., 1996

STM2383	*mepA*	Penicillin-insensitive murein DD-endopeptidase	-0.27	-0.06	
STM2439	*yfeL*	Putative membrane carboxypeptidase	0.06	-0.13	
STM3186	*tolC*	Required for detergent resistance and LPS cross-linking	0.06	0.10	
STM3197	*glgS*	RpoS dependent glycogen biosynthesis protein	-0.14	-0.17	
STM3200	*rfaE*	putative sugar nucleotide Transferase domain of ADP-L-glycero-D-manno-heptose synthase	-0.07	-0.15	Kim, 2003
STM3300	*dacB*	D-alanyl-D-alanine carboxypeptidase; penicillin-binding protein 4	-0.18	0.15	Baquero et al., 1996
STM3307	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	-0.20	-0.04	Kong et al., 2008
STM3308	*yrbA*	Putative BolA family transcriptional regulator	0.22	-0.20	
STM3309	*yrbB*	Putative STAS domain	-0.26	-0.24	
STM3310	*yrbC*	putative ABC superfamily transport protein	-0.34	-0.17	
STM3311	*yrbD*	Putative RBS for *yrbD*	-0.30	-0.07	
STM3312	*yrbE*	Putative ABC superfamily transport protein	-0.01	-0.15	
STM3313	*yrbF*	Putative ABC superfamily transport protein	0.15	0.02	
STM3314	*yrbG*	Putative CacA family Na:Ca transport protein	0.14	-0.03	
STM3315	*yrbH*	Putative polysialic acid capsule expression protein	-0.37	0.02	
STM3316	*yrbI*	Putative protein of HAD superfamily	-0.38	-0.04	
STM3317	*yrbK*	Putative inner membrane protein	-0.35	0.00	
STM3318	*yhbN*	Putative ABC superfamily transport protein	-0.11	-0.05	
STM3319	*yhbG*	Putative ABC superfamily transport protein	-0.11	-0.02	
STM3372	*mreD*	Rod shape-determining protein	-0.14	-0.01	Costa et al., 1993
STM3373	*mreC*	Rod shape-determining protein	-0.32	-0.25	Costa et al., 1993
STM3374	*mreB*	Rod shape-determining protein	-0.22	0.03	Costa et al., 1993
STM3493	*mrcA*	Transpeptidase of penicillin-Binding protein 1a; peptidoglycan synthetase	-0.15	-1.22	
STM3710	*rfaD*	ADP-L-glycero-D-mannoheptose-6-epimerase	-0.03	-0.04	Sirisena et al., 1994
STM3711	*rfaF*	LPS heptosyltransferase 1	-0.28	-0.12	Chen et al., 1993
STM3712	*rfaC*	Lipopolysaccharide heptosyltransferase-1	0.07	-0.13	Klena et al., 1992
STM3713	*rfaL*	O-antigen ligase	-0.17	-0.03	Klena et al., 1992

STM3714	*rfaK*	Lipopolysaccharide core biosynthesis	-0.27	0.00	Klena et al., 1992
STM3715	*rfaZ*	Lipopolysaccharide core biosynthesis protein	0.24	0.05	Klena et al., 1992
STM3716	*rfaY*	Lipopolysaccharide core biosynthesis protein	0.38	-0.06	Klena et al., 1992
STM3717	*rfaJ*	UDP-D-Glucose:(galactosyl)lipopolysaccharide glucosyltransferase	0.16	0.10	Pradel et al., 1992
STM3718	*rfaI*	UDP-D-Galactose:(glucosyl)lipopolysaccharide-alpha-1, 3-D-galactosyltransferase	0.31	-0.07	Pradel et al., 1992
STM3719	*rfaB*	UDP-D-Galactose:(glucosyl)lipopolysaccharide-1, 6-D-galactosyltransferase	-0.81	0.14	Pradel et al., 1992
STM3720	*yibR*	Putative inner membrane protein	-0.07	0.05	
STM3721	*rfaP*	Lipopolysaccharide core biosynthesis protein	-0.18	0.02	Parker et al., 1992
STM3722	*rfaG*	Glucosyltransferase I	-0.08	-0.12	Parker et al., 1992
STM3723	*rfaQ*	Lipopolysaccharide core biosynthesis protein	-0.27	-0.01	Parker et al., 1992
STM3724	*kdtA*	KDO transferase	-0.05	-0.01	Belunis et al., 1995
STM3725	*kdtB*	Phosphopantetheine adenylyltransferase	-0.05	-0.15	
STM3918	*rfe*	Undecaprenyl-phosphate N-acetylglucosaminyltransferase	-0.10	-0.25	Keenleyside et al., 1994
STM3919	*wzzE*	Ortholog of *E. coli* putative transport protein	-0.46	-0.47	Morona et al., 1995
STM3920	*wecB*	UDP-N-acetyl glucosamine-2-epimerase	-0.28	-0.19	Barua et al., 2002
STM3921	*wecC*	Ortholog of *E. coli* UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase	-0.12	-0.15	Bulach et al., 2000
STM3924	*wecD*	Lipopolysaccharide biosynthesis protein	-0.14	-0.21	Hung et al., 2006 Ramos-Morales et al., 2003
STM3925	*wecE*	TDP-4-oxo-6-deoxy-D-glucose transaminase	-0.09	-0.05	
STM3926	*wzxE*	O-antigen translocase in LPS biosyntesis	0.16	-0.26	
STM3927		putative inner membrane protein	-0.25	-0.31	
STM3928	*wecF*	*S.* Typhimurium probable 4-alpha-L-fucosyltransferase	-0.19	-0.33	
STM3929	*wecG*	*S.* Typhimurium probable UDP-N-acetyl-D-mannosaminuronic acid transferase	-0.08	-0.35	

STM3977	*rfaH*	Affecting biosynthesis of lipopolysaccharide core, F pilin, and haemolysin	-0.26	-0.20	Kong et al., 2009
STM4045	*rhaD*	Rhamnulose-1-phosphate aldolase	-0.42	-0.16	Al-Zarban et al., 1984
STM4046	*rhaA*	L-rhamnose isomerase	-0.20	0.10	Al-Zarban et al., 1984
STM4047	*rhaB*	Rhamnulokinase	0.29	0.02	Al-Zarban et al., 1984
STM4048	*rhaS*	AraC/XylS family; L-rhamnose operon regulatory protein	0.20	-0.14	Al-Zarban et al., 1984
STM4049	*rhaR*	AraC/XylS family; L-rhamnose operon transcriptional activator	-0.39	0.07	Al-Zarban et al., 1984; Tate et al., 1992
STM4050	*rhaT*	L-rhamnose-proton symport (L-rhamnose-H^+ transport protein)	-0.13	-1.00	Al-Zarban et al., 1984; Tate et al., 1992
STM4582	*slt*	Soluble lytic murein transglycosylase	-0.41	-0.03	

[a] PCN medium contains 0.4 mM Pi.

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10. PUBLICATION LISTS

PUBLICATION

Chen, PM, **HC Chen**, CT Ho, CJ Jung, HT Lien, JY Chen, and JS Chia. (2008) The two-component system ScnRK of *Streptococcus mutans* affects hydrogen peroxide resistance and murine macrophage killing. *Microbes and Infection.* **10**:293-301.

ORAL PRESENTATION

Chen HC, A Thompson, P Schwerk and K Tedin. (2010). A novel orphan *phoP/Q*-independent regulator influences *Salmonella* intracellular survival and antimicrobial peptides resistance. *National Symposium on Zoonoses Research. Berlin, Germany.*

POSTER PRESENTATIONS

Chen HC, A Thompson, P Schwerk and K Tedin. (2010) Identification and characterization of a Novel regulator STM0029 which contributes to *Salmonella* intracellular survival and resistance to antimicrobial peptides. *The Spetsai Summer course on "Host-Microbes Interactions". Spetses, Greece.*

Chen HC, D Chikkaballi, P Schwerk and K Tedin. (2010) A novel *Salmonella* gene involved in regulation of expression of the SPI4-encoded adhesin, SiiE. *3rd Joint Conference German Society for Hygiene and Microbiology. Association for General and Applied Microbiology. Hannover, Germany.*

Chen HC, D Chikkaballi, P Schwerk, and K Tedin. (2009) A novel gene involved in co-regulation of *Salmonella* pathogenicity islands 1 and 4. *Host Genetics Control of Infectious Diseases, Institut Pasteur, France.*

Chen HC, D Chikkaballi, P Schwerk, and K Tedin. (2008) Identification of the function of *yciGFE* operon is required for *Salmonella* invasion. *7th Louis Pasteur Conference on Infectious Diseases, France.*

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Datum/ Ort Unterschrift

11. SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, Heng-Chang Chen, dass ich die vorliegende Doktorarbeit mit dem Thema: „**Identification and Characterization of A Novel Salmonella Gene Product, STM0029, which Contributes to the Resistance to Host Antimicrobial Peptide Killing**“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

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Datum/Ort Unterschrift